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**MUTATED RAS PEPTIDES FOR GENERATION  
OF CD8<sup>+</sup> CYTOTOXIC T LYMPHOCYTES**

**FIELD OF THE INVENTION**

5 The present invention relates to mutated *ras* peptides and their use in the generation of human antigen specific cytotoxic T lymphocytes for prevention or treatment of cancer.

**BACKGROUND OF THE INVENTION**

10 Cancers in humans are commonly associated with mutations in dominant and recessive oncogenes. These genes produce mutated proteins that are unique to cancer cells. *Ras* proto-oncogenes are the best characterized mutated genes in human cancer (22-26). They encode a highly conserved family of 21 Kd proteins (p21). With a single amino acid mutation, the *ras* protein can potentiate transforming capabilities both in mouse and human cells. Such point mutated *ras* 15 have been found in a broad spectrum of human carcinomas notably at codons 12, 13 and 61. Codon 12 mutations form more than 90% of all *ras* mutations in human cancers.

20 Point mutations in the *ras* p21 proto-oncogenes (i.e., *K-ras*, *H-ras*, *N-ras*) have been identified, described and associated with a high frequency and spectrum of human cancers, including adenocarcinomas of the pancreas, colon and lung as well as melanomas and myeloid leukemias (reviewed in References 1-4). Such mutations lead to the production of aberrant proteins, which are distinct from normal endogenous *ras* p21 in both structure (DNA and protein sequences) and 25 function and represent early events in cellular transformation. In human carcinomas which harbor p21 point mutations, it is the *K-ras* gene at codon 12 that is found frequently mutated, whereby the normal glycine (Gly) residue is replaced with either an aspartic acid (Asp), valine (Val), cysteine (Cys), alanine (Ala), 30 arginine (Arg) or serine (Ser) residue (1,2,4). Substitutions of Gly to Asp, Val and Cys, collectively, however, account for the majority of human carcinomas with such p21 position 12 point mutations (1,2,4).

35 T Lymphocytes can recognize antigens (Ag) presented in the context of major histocompatibility complex (MHC) class I or class II molecules on the

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surface of antigen presenting cells (APC) (27-31). These surface Ag are thought to be short peptides that are derived from degraded intact proteins (32-34).

T lymphocytes are divided into two major populations, CD4<sup>+</sup> and CD8<sup>+</sup>. The CD8<sup>+</sup> T cells recognize peptides (8-10 residues) bound to MHC class I molecules and are associated with cytotoxic activity (30-31). The CD4<sup>+</sup> lymphocytes are generally involved in the recognition of peptides (13 - 18 residues) presented in the context of MHC class II molecules (28,29), and are involved in immunoregulation through cytokine secretion. Th1, a subset of CD4<sup>+</sup> T cells have been reported to express lytic activity (35-37).

Data in mice has shown the generation of MHC restricted (specific) cytotoxic T cells that are capable of detecting endogenous, cytoplasmic peptide antigens (antigens presented from within the cell membrane on the cell surface) <sup>38,39</sup>. These T cells can cause rejection (lysis) of cells expressing such peptides. This rejection is mediated by cells responding to novel peptides derived from mutated genes (38,39). T cells are capable of detecting single amino acid discrepancies between homologous peptides presented on APC (40,41).

The generation and expression of these previously unseen, "neo-determinants" may now represent unique and highly specific epitopes for T cell (CD4<sup>+</sup> and/or CD8<sup>+</sup>) recognition, which has been proposed in host defense as an important effector pathway in the control of malignancy (5). Studies in both murine and human systems, have previously identified and characterized immunodominant CD4<sup>+</sup> T cell epitopes of mutant *K-ras* at codon 12 using short synthetic peptides (6-12). With respect to human anti-*ras* CD8<sup>+</sup> cytotoxic T lymphocyte (CTL) responses, however, nothing is yet defined for position 12 mutations, although one has been reported for position 13 and one for position 61 (13, 14). Thus, the identification herein of human CD8<sup>+</sup> T cell epitopes reflecting specific *ras* p21 point mutations has important and direct implications for the development of oncogene-specific vaccines in cancer immunotherapy.

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### SUMMARY OF THE INVENTION

The invention is mutant *ras* peptides and variants or analogs thereof which elicit antigen specific cytotoxic T lymphocytes. The mutant *ras* peptides are T cell epitopes for CD8<sup>+</sup> human T lymphocytes.

Another aspect of the invention is a pharmaceutical composition comprising one or more mutant *ras* peptides and a pharmaceutically acceptable carrier. The pharmaceutical composition is useful as an immunogen and as a therapeutic in the prevention or treatment of cancer and in inhibiting growth of tumors expressing a *ras* mutation. The pharmaceutical composition may further comprise an adjuvant or a liposome formulation.

Another pharmaceutical composition comprises a mutant *ras* peptide-pulsed antigen presenting cell and a pharmaceutically acceptable carrier. The composition is useful as an immunogen and as a therapeutic.

A further object of the invention is a mutant *ras* p21 protein or peptide specific cytotoxic T lymphocyte which prevents the occurrence of tumor cells and inhibits the growth of or kills tumor cells which express the mutant *ras* p21 protein or peptide.

Another aspect of the invention is a method of generating cytotoxic T lymphocytes specific against tumors expressing a *ras* mutation by *in vivo* administration of an effective amount of at least one mutant *ras* peptide alone, or in combination with an adjuvant, in a liposome formulation, or by administration of mutant *ras* peptide-pulsed antigen presenting cells. The antigen specific cytotoxic T lymphocytes which arise from immunization are useful in methods of inhibiting or killing tumor cells expressing a mutant *ras* p21 protein or peptide.

Yet another aspect of the invention is a method of generating cytotoxic T lymphocytes specific against tumors expressing a *ras* mutation *in vitro* by stimulation of lymphocytes from a source with an effective amount of a mutant *ras* peptide, alone or in combination with one or more cytokines. Such antigen specific cytotoxic T lymphocytes may be adoptively transferred into a mammal for the prevention or treatment of cancer and to inhibit or kill tumors expressing the

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- ° mutant *ras* protein, or peptide. Further, the cytotoxic T lymphocytes are useful in methods for screening for antigen epitope mapping.

A further aspect of the invention is a method of preventing the occurrence, inhibiting the growth of or killing tumor cells expressing mutant *ras* p21 protein or peptides comprising a) generating mutant *ras* protein or peptide specific cytotoxic T lymphocytes *in vitro* by stimulation of lymphocytes from a source with an effective amount of a mutant *ras* peptide, alone or in combination with one or more cytokines, the amount being effective in generating mutant *ras* protein or peptide specific cytotoxic T lymphocytes, and b) adoptively transferring the mutant *ras* protein or peptide specific cytotoxic T lymphocytes alone, or in combination with one or more cytokines into a mammal in an amount sufficient to prevent the occurrence of, inhibit the growth of, or kill the tumor cells.

Another aspect of the invention is a method of preventing the occurrence, inhibiting the growth or killing tumor cells expressing mutant *ras* p21 protein or peptide in a mammal comprising a) generating mutant *ras* p21 protein or peptide specific cytotoxic T lymphocytes *in vivo* by administration of an effective amount of a mutant *ras* protein alone, or in combination with an adjuvant or as a liposome formulation, and b) the mutant *ras* p21 protein or peptide specific cytotoxic T lymphocytes so generated prevent the occurrence of, inhibit the growth of, or kill the tumor cells in the mammal.

Another object of the invention is a DNA sequence encoding one or more mutant *ras* peptides.

Another object of the invention is a vector comprising at least one insertion site containing a DNA sequence encoding one or more mutant *ras* peptides, operably linked to a promoter capable of expression in a host cell.

Yet another object of the invention is a method of generating mutant *ras* peptide specific cytotoxic T lymphocytes by administration into a mammalian host an effective amount of a recombinant virus vector comprising at least one insertion site containing a DNA sequence encoding a mutant *ras* peptide.

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### DESCRIPTION OF THE DRAWINGS

*Figure 1A and 1B.* Generation of a peptide-specific CD4<sup>+</sup> T cell line from immune lymphocytes of RAS patient 43. Following the third vaccination, a CD4<sup>+</sup> T cell line from RAS patient 43 was established and maintained in culture by continuous weekly IVS of unfractionated immune lymphocytes with autologous PBMC (up to cycle 3) or BLCL (thereafter) as APC incubated with the mutant *ras* 13-mer peptide and IL-2. Demonstration of Ag specificity and potency of the T cell culture was examined by lymphoproliferation, and the results expressed as stimulation index. (Figure 1A) Proliferative response measured weekly starting at IVS cycle 3; and (Figure 1B) Proliferative response at IVS cycle 6 against specific and irrelevant peptides. Phenotypic analysis revealed the T cell culture > 85% CD4<sup>+</sup>.

*Figure 2A and 2B.* Proliferation Response by RAS 43 T Cell Line is Mediated by CD4<sup>+</sup> Lymphocytes Which are MHC class II-Restricted. (Figure 2A) MAb directed against HLA class I (i.e., anti-HLA-A,B,C) or class II (i.e., anti-HLA-DR, DP or DQ) molecules were used to define the role and importance of MHC restriction for T cell activation. The T cell line of RAS patient 43 was assayed after IVS cycle 10 using autologous BLCL as APC, incubated with peptide (10 µg/ml) ± MAb at different concentrations. Results are expressed as % control, as defined by the equation: [(activity with MAb<sub>cpm</sub> - control<sub>cpm</sub>)/(activity without MAb<sub>cpm</sub> - control<sub>cpm</sub>)] x 100, where control<sub>cpm</sub> refers to unstimulated cultures of T cells+BLCL without peptide). (Figure 2B) In a separate experiment, MAb directed against the CD4 (clone OKT4; hybridoma supernatant, 20% v/v) or CD8 (clone OKT8; hybridoma supernatant, 20% v/v) molecules were used to identify and confirm the functional T cell subset(s). Also included in this assay were MAb directed against HLA class I or II molecules (each at 3 µg/ml), as in Figure 2A. In Figure 2B, the T cell line was tested after IVS cycle 15 using autologous BLCL as APC, incubated with Cys12 peptide (10 µg/ml) ± MAb. Additional control cultures (cpm ± SEM) included: irradiated BLCL, 8,520 ± 225; T cells+BLCL+Gly12 peptide (10 µg/ml), 7,690 ± 383).

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Figure 3. Generation of a peptide-specific CD8<sup>+</sup> T cell line from immune lymphocytes of RAS patient 32. After the third vaccination, PBMC of RAS patient 32 were enriched in CD8<sup>+</sup> (46.9%; CD4<sup>+</sup>, 2.2%) T cells. Cultures were initiated and maintained by continuous weekly IVS cycles using autologous PBMC (up to cycle 3) or BLCL (thereafter) as APC incubated with mutant *ras* peptide plus  $\beta_2$ -microglobulin and IL-2. The mutant *ras* peptide represented a nested HLA-A2 binding, 10-mer sequence [i.e., *ras*5-14(D12)]. Cytotoxicity was determined weekly by a standard 6 hr <sup>51</sup>Cr-release assay using the T2 cell line as a target, incubated with mutant or normal *ras* peptide (i.e., 10-mer sequences @ 10  $\mu$ g/ml), as shown. Results illustrated at E/T ratios of 10/1 and 5/1.

Figure 4. Cytolytic activity by CD8<sup>+</sup> CTL line of RAS patient 32 is HLA-A2-restricted. The CD8<sup>+</sup> CTL line of RAS patient 32 (see Fig. 3) was assayed against a panel of HLA-A2<sup>+</sup> targets, with and without peptide (10  $\mu$ g/ml). Cytotoxicity was determined by <sup>51</sup>Cr-release, and the results expressed at E/T ratio of 20/1 following IVS cycle 16. Similar results were observed using a second, independently-derived CD8<sup>+</sup> CTL line as described in Table 6. Also, using this second CD8<sup>+</sup> CTL line, anti-HLA-A2 MAb (clone BB7.2) inhibited peptide-specific cytotoxicity against C1R-A2 targets (not shown).

Figure 5. Cytolytic activity by CD8<sup>+</sup> CTL line of RAS patient 32 is peptide-specific. The CD8<sup>+</sup> CTL line of RAS patient 32 (see Fig. 3) was assayed against the T2 cell line as a target, incubated with and without different *ras* peptides (10  $\mu$ g/ml), as shown. In parallel, the extent of NK activity was evaluated against K562 cells. Cytotoxicity was determined by <sup>51</sup>Cr-release, and the results expressed at different E/T ratios following IVS cycle 7. Similar results were observed using the second, independently-derived CD8<sup>+</sup> CTL line as described in Table 6.

Figure 6. Generation of a CD8<sup>+</sup> T-Cell line specific for the mutant *ras*4-12(V12) peptide. A CD8<sup>+</sup> CTL line specific for *ras*4-12(V12) was initiated from a normal HLA-A2<sup>+</sup> donor using a model antigen presentation system, consisting of the T2 cell line (expressing heightened levels of B7.1), peptide,  $\beta_2$ -microglobulin and cytokines (IL-2, IL-12). Autologous Epstein Barr Virus-transformed B-cells

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replaced T2 cells as antigen presenting cells after the second IVS cycle. Here, the importance of both peptide specificity and HLA restriction for cytotoxicity was examined against targets either expressing HLA-A2 (i.e. allogeneic B-cell line matched for HLA-A2 or the melanoma line, MEL-624) or lacking HLA-A2 (i.e. allogeneic B-cell line mismatched for HLA-A2). Cytotoxicity was determined after IVS cycle 6, in the absence or presence of mutant or normal *ras* peptide (i.e. 9-mer sequences @ 5  $\mu$ g/ml) as shown. Results illustrated at effector/target ratio of 20/1 and expressed as mean  $\pm$  SEM of triplicate wells.

*Figure 7.* Cytolytic activity by CD8<sup>+</sup>CTL line of patient 32 is HLA-A2-restricted. The requirement for HLA-A2 in peptide presentation (by C1R-A2 targets) was determined in blocking experiments using MAb (clone BB7.2; ascites = 1:100 dilution) directed against that molecule (or an isotype-matched MAb). IVS cycle = 16; effector/target ratio = 10/1; *ras*5-14(Asp12)peptide = 1  $\mu$ g/ml. MAb directed against the CD4 (clone OKT4; hybridoma supernatant, 20% v/v) or CD8 (clone OKT8; hybridoma supernatant, 20% v/v) molecules were used to identify and confirm the functional T cell subset(s). Control lysis in the absence of peptide or in the presence of *ras*5-14(Gly12) was <3%.

*Figure 8A and 8B.* Production of a peptide-specific CD4<sup>+</sup> T cell line from post-vaccinated lymphocytes of patient 29. Fig. 8A: Unfractionated PBMC, pre-vaccination and post-third vaccination, were stimulated biweekly using autologous PBMC as APC incubated with mutant *ras* 13-mer peptide [i.e., *ras*5-17(Val12)] and IL-2, similar to patient 43 (see Table 3). PBMC cultures were following the second IVS. Proliferation was measured by <sup>3</sup>H-thymidine uptake and the results were expressed as stimulation index (SI). Fig. 8B: MAb directed against the CD4 (clone OKT4; hybridoma supernatant, 20% v/v) or CD8 (clone OKT8; hybridoma supernatant, 20% v/v) molecules were used to identify and confirm the functional T cell subset(s). The T cell line of patient 29 was assayed after IVS cycle 4 using autologous EBV-B cells as APC, incubated with *ras*5-17(Val12) peptide (3  $\mu$ g/ml)  $\pm$  MAb. Additional control cultures (cpm  $\pm$  SEM) included: irradiated EBV-B cells (3,535  $\pm$  372); T cells+EBV-B cells (19,256  $\pm$  1926); T cells+EBV-B cells+Gly12 peptide (3  $\mu$ g/ml), (18,116  $\pm$  1474).

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Figure 9A and 9B. Production of a peptide-specific CD8<sup>+</sup> T cell line from post-vaccinated lymphocytes of patient 29. Figure 9A: A CD8<sup>+</sup> CTL line was established from post-vaccinated PBMC of patient 29 and assayed for peptide specificity against C1R-A2 targets. Cytotoxicity was determined by <sup>51</sup>Cr-release, and the results expressed at different effector/target ratios following IVS cycle 7. Figure 9B: Using this CD8<sup>+</sup> CTL line, the requirement for HLA-A2 in peptide presentation (by autologous EBV-B cells as targets) was determined in blocking experiments using MAb (clone BB7.2; ascites = 1:30 dilution) directed against that molecule (or using an isotype-matched MAb). IVS cycle = 11; effector/target ratio = 10/1; *ras*5-14(Val12) peptide = 3 μg/ml. CD4 and CD8 MAbs were as described for Figure 8A and 8b. Control lysis in the absence of peptide or in the presence of *ras*5-14(Gly12) was <3%.

Figure 10A and 10B. Anti-*ras* Val12-specific CTL from patient 29 lyse SW480 tumor targets in the absence of exogenous peptide. Figure 10A: Anti-*ras* Val12-specific CD8<sup>+</sup> CTL from patient 29 were assayed against the SW480 colon carcinoma cell line (HLA-A2<sup>+</sup>, *ras*Val12+) in the absence of exogenous peptide, with or without IFN-γ pretreatment (250 U/ml for 24 hr) of the target cells. CD8<sup>+</sup> CTL reactive with the MART-1<sub>27-25</sub> peptide were used as an irrelevant effector cell population. Cytotoxicity was determined by <sup>51</sup>Cr-release, and the results expressed at different effector/target ratios. Figure 10B: In parallel to 10A, anti-*ras* or anti-MART-1-specific CTL were assayed against SW480 (±IFN-γ pretreatment) at an effector/target ratio of 10/1 in the absence or presence of exogenous peptide (5 μg/ml). For anti-*ras* CTL: specific peptide = *ras*5-14(Val12); control peptide = *ras*5-14(Gly12). For anti-MART-1 CTL: specific peptide = MART-1<sub>27-35</sub>; control peptide = *ras*5-14(Val12). Flow cytometric analysis of SW480 cells for expression of HLA-A2: untreated = 65.4% with MFI of 29.8; IFN-γ-pretreated = 99.5% with MFI of 349.6. Flow cytometric analysis of SW480 cells for expression of ICAM-1: untreated = 16.0% with MFI of 30.3; IFN-γ-pretreated = 98.6% with MFI of 181.9.

Figure 11. Identification of a Human Mutant *ras* CD8<sup>+</sup> CTL Peptide Epitope Variant that Enhances Effector Function. The CD8<sup>+</sup> CTL line of patient



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- 32 was assayed against the C1R-A2 cell line as a target, incubated with the different *ras* peptides at several concentrations, as shown. Cytotoxicity was determined by a standard 6 hour <sup>51</sup>Cr-release, and the results expressed at an effector/target ratio of 10/1. Results show that the introduction of a N-terminal Tyr residue (Y5) in mutant *ras* peptide 5-14(Asp12) enhances CTL activity against peptide-pulsed targets.

#### **DETAILED DESCRIPTION OF THE INVENTION**

The invention is a mutated *ras* peptide, analog or variant thereof. The mutated *ras* peptide is characterized by its ability to elicit an immune response specific against mutant *ras*-p21 protein or portion thereof and against cells expressing or binding mutant *ras*-p21 protein or portion thereof.

The mutant *ras* peptide of the present invention elicits antigen specific cytotoxic T lymphocytes which inhibit the growth or kill cells expressing mutant *ras*-p21 protein or peptides thereof.

Cells that express mutant *ras*-p21 protein or peptides thereof include but are not limited to cancer cells, in particular, human cancer cells. Such cancers include, but are not limited to, adenocarcinomas of the pancreas, colon, endometrial, lung, thyroid, melanoma, oral laryngeal, seminoma, hepatocellular, bile duct, acute myeloblastic leukemia, basal cell carcinoma, squamous cell carcinoma and the like.

Of particular interest are cancers with position 12 mutations in the *ras* p21 protein or peptide.

Three *ras* genes with transforming potential include H-, K- and N- *ras*. H-, K- and N- *ras* p21 proteins share an overall 75% amino acid homology. Within the N-terminal catalytic domains (positions 5 to 120) the homology between the three oncogenes is greater than 97%. Thus, the pointed mutated *ras* peptides of the present invention are effective in treating cancers induced by one or more *ras* oncogenes. Such cells expressing mutant *ras*-p21 protein or peptides may be inhibited from growing or killed by mutant *ras*-p21 peptide specific cytotoxic T lymphocytes or CD8<sup>+</sup> lymphocytes, both *in vitro* and *in vivo*.

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### Amino Acid and Position

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Mutant *ras5-14*  
(Asp12) Lys-Leu-Val-Val-Val-Gly-Ala-~~Asp~~-Gly-Val (SEQ. ID NO. 3)

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Mutant *ras5-14*  
(Cys12) Lys-Leu-Val-Val-Val-Gly-Ala-Cys-Gly-Val (SEQ. ID NO. 5)

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or variants or analogs thereof. Variants or analogs may be constructed that elicit a more potent T-cell response.

Analogues are constructed for enhanced binding to MHC class I molecules and/or enhanced binding to the T cell receptor for an improved immune response.

5 Variants or analogs may include but are not limited to peptides having a single amino acid substitution at a position distinct from position 12. Such substitutions include but are not limited to changing Lys at position 5 with Tyr. In one such embodiment, a variant of SEQ ID NO.: 3 includes Tyr-Leu-Val-Val-Val-Gly-Ala-Asp-Gly-Val (SEQ. ID NO.: 11).

10 The mutant *ras* peptide may be obtained by recombinant DNA technology, by chemical peptide synthesis or by appropriate protease cleavage of an isolated, mutant *ras* protein or peptide.

15 The mutant *ras* peptide may be formulated into a pharmaceutical composition in combination with a pharmaceutically acceptable carrier for use as an immunogen in a mammal, preferably a human. The composition may further comprise one or more other constituents to enhance the immune response which include but are not limited to biological response modifiers such as interleukin 2, 20 interleukin 6, interleukin 12, interferon, tumor necrosis factor, GM-CSF, and cyclophosphamide.

25 The mutant *ras* peptide is administered to a mammal in an amount effective in generating a mutant *ras* peptide specific immune response, preferably a cellular immune response. The efficacy of the mutant *ras* peptide as an immunogen may be determined by *in vivo* or *in vitro* parameters as are known in the art. These parameters include but are not limited to antigen specific cytotoxicity assays, regression of *Ras*-p21<sup>+</sup> tumors, inhibition of *Ras*-p21<sup>+</sup> cancer cells, production of cytokines and the like.

30 At least one or more mutant *ras* peptides may be administered in a dose of about 0.05 mg to about 10 mg per vaccination of the mammal, preferably about 0.1 mg to about 5 mg per vaccination. Several doses may be provided over a period of weeks as indicated. In one embodiment a dose is provided every month for 3 months. The mutant *ras* peptide may be administered alone or in

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combination with adjuvants, in a liposome formulation, cytokines, biological response modifiers, or other reagents in the art that are known to enhance immune response. Adjuvants include but are not limited to RIBI Detox™, QS21, alum and incomplete Freund's adjuvant. In one embodiment, the mutant *ras* peptide is administered in combination with Detox™ (RIBI Immunochem, Hamilton, MT).

The mutant *ras* peptides may also be conjugated to helper peptides or to large carrier molecules to enhance the immunogenicity of the peptide. These molecules include but are not limited to influenza peptide, tetanus toxoid, tetanus toxoid CD4 epitope, Pseudomonas exotoxin A, poly-L-lysine, and the like.

Another effective form of the mutant *ras* peptide for generating a mutant *ras* peptide specific immune response in a mammal is a mutant *ras* peptide-pulsed antigen presenting cell. The antigen presenting cells include but is not limited to dendritic cells, B lymphocytes, monocytes, macrophages and the like. In a preferred embodiment, the mutant *ras* peptide-pulsed antigen presenting cell is a dendritic cell.

The invention also provides a method of generating mutant *ras* peptide specific cytotoxic T lymphocytes *in vivo* or *in vitro* by stimulation of lymphocytes from a source with an effective amount of a mutant *ras* peptide alone or in combination with a biological response modifier and/or adjuvant or in a liposome formulation. The sources of lymphocytes include but are not limited to peripheral blood, tumor tissues, lymph nodes and effusions such as pleural fluid or ascites fluid and the like.

The mutant *ras* p21 protein or peptide specific cytotoxic T lymphocytes of the present invention are immunoreactive with mutant *ras* p21 protein or peptide. The cytotoxic T lymphocytes inhibit the occurrence of tumor cells and cancer and inhibit the growth or kill mutant *ras* p21 protein or peptide expressing tumor cells. The cytotoxic T lymphocytes, in addition to being antigen specific, are MHC class I restricted. In one embodiment the cytotoxic T lymphocytes are MHC class I HLA-A2 restricted. The cytotoxic T lymphocytes have a CD8<sup>+</sup> phenotype. The cytotoxic T lymphocytes may be restricted by other HLA alleles including but not limited to A3, A11, A68, A24 and the like.

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° A peptide-based, phase I clinical trial was initiated in metastatic carcinoma patients whose tumors contain point mutations in the *ras* p21 proto-oncogenes at codon 12. The majority of patients in this study presented primary malignancies of the colon, GI tract, lung and pancreas and harboring position 12 mutations of Gly to Asp, Cys or Val.

5 Selected patients were vaccinated subcutaneously up to three times at monthly intervals with DETOX™ adjuvant admixed with the appropriate mutated *ras* 13-mer peptide spanning positions 5-17, which corresponded to the specific point mutation at codon 12 found in their cancer. Recently, Gjertsen et al. (15) 10 reported on the vaccination of pancreatic carcinoma patients with autologous peripheral blood mononuclear cells pre-pulsed ex vivo with mutant *ras* peptides (spanning positions 5-21) reflecting codon 12 mutations. Of five patients receiving multiple vaccinations, two patients showed evidence of anti-*ras* T cell responses, as 15 measured by proliferation assays. In one patient, the T cell response was specific for the immunizing peptide (Val12); whereas, in the other patient, the T cell response cross-reacted with both immunizing (Asp12) and non-mutated (Gly12) peptides. In both patients, these responses were transient in that they were 20 detectable at day 40 after the onset of vaccination, but undetectable during the following weeks. No other phenotypic or functional studies were reported.

In the present invention, the capacity to induce peptide-specific cellular immune responses in patients vaccinated with mutant *ras* peptides was shown. Moreover, the present invention provides for the first time of a human HLA-A2- 25 restricted, CD8<sup>+</sup> CTL epitope reflecting the codon 12 mutation, Gly to Asp. Major histocompatibility complex (MHC) class II-restricted CD4<sup>+</sup> T cell lines were derived from individual patients. In addition, MHC class I-restricted CD8<sup>+</sup> T cell line was produced, which recognized a nested sequence of the original 30 immunogen. Both CD4<sup>+</sup> and CD8<sup>+</sup> T cell lines could be maintained long term in culture without loss of antigen (Ag) specificity. Furthermore, in the patients, no specific T cell responses were found against the normal *ras* sequence and no T cell lines were generated in culture from pre-immune lymphocytes. Taken collectively, 35 vaccination with oncogene-derived mutant *ras* peptides of the present invention

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induces highly specific and systemic anti-*ras* cellular immune responses. Moreover, the development of such MHC class I-restricted mutant *ras* peptides has important implications for both active (i.e., vaccination) and passive (i.e., ex vivo expansion for cellular adoptive transfer) immunotherapies, which may be used for the induction and propagation of specific CD8<sup>+</sup> CTL responses in cancer patients.

In accordance with the present invention, patient immune status was compared pre-vaccination and postthird vaccination. The phenotypic and functional properties of the resulting T cell lines established from those patients which displayed evidence of cell-mediated immunity were characterized. The capacity to induce peptide-specific CD4<sup>+</sup> T cell responses in a subset of patients following vaccination with *ras* oncogene peptides reflecting the corresponding *ras* mutation was confirmed. Moreover, the present invention provides for the first time the identification of human HLA-A2-restricted, CD8<sup>+</sup> CTL epitopes reflecting two distinct codon 12 mutations, which were found to be nested within the longer 13-mer peptide immunogen (i.e., *ras*5-14(D12)). Thus, in an aspect of the present invention, it was demonstrated that a single mutant *ras* peptide immunogen contained both CD4<sup>+</sup> and CD8<sup>+</sup> T cell epitopes in a nested configuration. Experimental models were established *in vitro*, which demonstrated the capacity of CD4<sup>+</sup> and CD8<sup>+</sup> T cell lines to recognize antigen presenting cell (APC) populations presenting the corresponding oncoprotein or tumor cells harboring the naturally-occurring mutation.

That a single mutant *ras* peptide immunogen contains both CD4<sup>+</sup> and CD8<sup>+</sup> T cell epitopes in an overlapping or nested configuration, as demonstrated by the present invention, affords important biological implications for the generation and coordination of a more efficient anti-pathogen immune response. Similarly, in a murine model, overlapping MHC class II-restricted CD4<sup>+</sup> and MHC class I-restricted CD8<sup>+</sup> CTL peptide epitopes were also identified, thus reflecting the *ras* Val12 mutation. In accordance with the present invention, oncogene-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell lines may be considered for employment in adoptive immunotherapy, perhaps in concert with active immunization for a more comprehensive antitumor attack. Furthermore, as supported by the present

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invention, modifications in immunogen design, schedule and delivery, along with the co-administration of cytokines such as IL-2, IL-12 or GM-CSF, are likely to enhance both the development of the immune response and potential clinical benefit.

5 In accordance with the present invention, methods were developed for improving the sensitivity and consistency for detecting weakly positive peptide-specific proliferative responses and functional evaluation of cultured lymphocyte populations. To this end, patient lymphocyte populations were incubated *in vitro* (at 7-14 day intervals) with an autologous source of APC, mutant *ras* peptide as antigen (Ag) and IL-2, and then were retested for peptide-specific reactivity at or toward the end of an IVS cycle. Such culture conditions were developed to reflect the nominal requirements for Ag-specific expansion of *in vivo* peptide-primed lymphocytes and their subsequent testing as evidence for "recall" responses to  
10 vaccination. These IVS cycles were established to: (i) amplify detection of a weak, but specific functional response; (ii) compare pre-vaccine to post-vaccine lymphocytes over a period of time for assessment of potential peptide-specific reactivity; and (iii) derive peptide-specific T cell lines in culture for detailed  
15 phenotypic and functional characterization.

According to the present invention, methods for the induction of CD8<sup>+</sup> CTL specific for position 12 mutations are provided. HLA-A2-restricted responses were identified in two of three vaccinated patients. This was made possible, in  
25 large part, by the initial identification of a nested peptide sequence bearing a consensus anchor motif for HLA-A2, which displayed functional binding to that molecule (Table 7). *In vivo* priming of the CD8<sup>+</sup> T cell response likely resulted from *in vitro* mechanisms of Ag processing (i.e., extracellular or intracellular), perhaps influenced and potentiated in the presence of adjuvant, with the subsequent  
30 generation of MHC class I-reactive epitopes. Although the CTL analysis was initially examined and demonstrated for HLA-A2/peptide interactions, the present invention provides vaccination-induced CTL responses restricted by other HLA class I alleles. Thus, insights into patient immune responsiveness to these

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immunogens may be provided by a detailed analysis of HLA restriction patterns for peptide presentation, and identification of putative consensus anchor motifs.

Patients with solid tumors expressing mutant *ras*, including but not limited to colon cancer, lung cancer, pancreas cancer, endometrial cancer, thyroid cancer, melanoma, oral cancer, laryngeal cancer, seminoma, hepatocellular cancer, bile duct cancer, acute myeloblastic leukemia, basal cell carcinoma, squamous cell carcinoma, prostate cancer and the like benefit from immunization with the mutant *ras* peptides. A tumor tissue sample is obtained from a patient for determination of the *ras* mutation using techniques known in the art such as PCR analysis. Patients amenable to treatment using the mutant *ras* peptides of the present invention are those patients having tumors with *ras* mutations in the normal *ras* p21 protein. Of particular interest are tumors with a point mutation in codons 12, in particular mutations resulting in Gly to Cys, Gly to Asp, Gly to Val, Gly to Ala, Gly to Arg and Gly to Ser at position 12.

The mutant *ras* peptide used for immunization is one which corresponds with the *ras* mutation in the tumor of the patient. The peptides may be chemically synthesized under GMP conditions and purified by HPLC to >95% purity and lyophilized. Pharmaceutical compositions are formulated by reconstituting the peptide with a pharmaceutically acceptable carrier such as sodium chloride. In one example, each milliliter of solution contains 1500  $\mu$ g of a mutant *ras* peptide plus 9.0 mg sodium chloride. The mutant *ras* peptides with Cys or Val at position 12 are formulated at pH = about 4.5 to about 6.5. The mutant *ras* peptide with Asp at position 12 are formulated at pH = about 3.5 to about 5.5.

When the mutant *ras* peptide is administered with an adjuvant it is desirable to mix the peptide with the adjuvant shortly before administration to a patient.

The mutant *ras* peptide may be administered to a patient by various routes including but not limited to subcutaneous, intramuscular, intradermal, intraperitoneal, intravenous and the like. In one embodiment the mutant *ras* peptide is administered subcutaneously. The peptide may be administered at one or more sites to a patient. In one embodiment, the peptide, alone or in combination

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with an adjuvant, is administered into three sites subcutaneously, over the deltoids, the thighs and the abdomen.

In another method of generating an immune response, mutant *ras* peptide-pulsed antigen presenting cells are administered to the patient in an amount effective to generate an antigen specific immune response. The antigen presenting cells include but are not limited to dendritic cells, B lymphocytes, monocytes, macrophages and the like. In one embodiment, dendritic cells are isolated from a patient by methods described in Romani, N. et al (1994). The isolated dendritic cells are cultured *in vitro* with a mutant *ras* peptide for a period of about 0.5 to about 3 hours and washed to remove non-bound peptide. The mutant *ras* peptide-pulsed dendritic cells are transferred back into the patient at a concentration of about  $10^6$  to about  $10^9$  dendritic cells. Such a concentration is effective in generating an immune response in the patient including the generation of mutant *ras* p21 protein or peptide specific cytotoxic T lymphocytes which are able to inhibit the growth or kill tumor cells.

The criteria for determining an anti-tumor response in the immunized patient is as follows:

1. Complete Remission (CR): Complete disappearance of all evidence of tumor and return of abnormal tests to normal levels for a minimum of 4 weeks.
2. Partial Response (PR): Decrease by at least 50% in the sum of the products of the perpendicular diameters of all measured lesions in the absence of progression of any lesion nor the appearance of any new lesions for at least 4 weeks.
3. Stable Disease (SD): Change in measurable disease too small to meet the requirements for partial response or progression and the appearance of no new lesions for a period of at least 12 weeks. There may be no worsening of symptoms.
4. Progressive Disease (PD) or Relapse: Any one of the criteria below must be met to be considered progressive disease:
  - Development of any new area of malignant disease (measurable or palpable),

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° Increase (>25%) in any pretreatment area of measurable malignant disease.

The immunological response to immunization with the mutant *ras* peptides are assessed by in-vitro T cell proliferation assay and/or by in-vitro T cell cytotoxic assay before and after vaccination.

In vitro T cell proliferation assay:

The patient PBMC are incubated *in vitro* with the appropriate tumor specific *ras* peptide, and evaluated for peptide-induced proliferation following up to 6 days of incubation. Cultures are pulsed with [<sup>3</sup>H]-thymidine for the final 18-24 hours of their culture. Proliferation is measured and quantified by the incorporation of [<sup>3</sup>H]-thymidine. A proliferation of more than three fold above control (i.e. without peptide stimulation or with the normal *ras* peptide) is considered as a positive response.

In vitro T cell cytotoxic assay:

The T cell cytotoxicity is measured by the standard [<sup>51</sup>Cr]-release assay. Briefly, target cells (autologous tumor cells or autologous EBV-transformed B cells) are radiolabeled with Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub>. The patient PBMC (which has previously stimulated with the appropriate *ras* peptide) is added to the labeled target cells in the presence or absence of the corresponding *ras* peptide. Cell lysis is determined by the specific release of <sup>51</sup>Cr (specific lysis). If there is a detectable pre-immunization specific lysis, a 1.5 fold increase in the lysis will be considered a positive response. If there is no detectable pre-immunization specific lysis, a post-immunization specific lysis of than 15% is considered a positive response.

The present invention includes *in vitro* immunization for T cell proliferation and generation of cytotoxic T cell lines to the tumor specific *ras* mutated peptide. *In vitro* cultivation of peptide specific T cells from peripheral blood mononuclear cells (PBMC), lymph node tissue (LNT), or tumor infiltrating lymphocytes (TIL) with mutant *ras* peptide and IL2 generates peptide specific T cells. These T cells are tested for cytotoxicity against mutant *ras* peptide primed APC (autologous EBV transformed B cells or autologous tumor cells) has described herein. Generated T cell clones are characterized phenotypically by flow cytometry for express of CD3,

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CD4, and CD8. Mutant *ras* peptide specific cytotoxic lymphocytes may be adoptively transferred to a patient in order to inhibit or kill mutant *ras* p21 expressing tumor cells. Patients may then be reimmunized with mutant *ras* peptide preferably in adjuvant.

Generally, between about  $1 \times 10^5$  and  $2 \times 10^{11}$  cytotoxic T cells per infusion are administered in, for example, one to three infusions of about 200 to about 250 ml each over a period of 30 to 60 minutes. After completion of the infusions, the patient may be treated with a biological response modifier such as interleukin 2 (IL-2). In the case of IL-2, recombinant IL-2 is administered intravenously in a dose of 720,000 IU per kilogram of body weight every eight hours. After adoptive transfer of the antigen specific cytotoxic T cells into the patient, the patient may be additionally treated with the mutant *ras* peptide used to prime the cytotoxic T cells, to further expand the T cell number *in vivo*.

The invention encompasses a DNA sequence and analogs thereof which encode a mutant *ras* peptide. The DNA sequence encoding the mutant *ras* peptide differs from the DNA sequence of the normal *ras* p21 protein or peptide by a point mutation in the DNA sequence. Of particular interest are DNA sequences comprising a point mutation at the codon encoding the amino acid at position 12. The normal codon encoding glycine at position 12, i.e. GGT, may be substituted by a mutant codon encoding aspartic acid, cysteine, valine, alanine, arginine, and serine. In a preferred embodiment, the normal codon is substituted by a mutant codon encoding aspartic acid, valine, or cysteine.

In one embodiment the DNA sequence encoding the mutant *ras* peptide comprises:

TAT	AAA	CTT	GTG	GTA	GTT	GGA
Tyr	Lys	Leu	Val	Val	Val	Gly
	5					10

GCT	GAT	GGC	GTA	GGC	AAG	AGT	(SEQ. ID NO. 7)
Ala	Asp	Gly	Val	Gly	Lys	Ser	(SEQ. ID NO. 12)
				15			

or portion or variant thereof.

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In another embodiment the DNA sequence encoding the mutant *ras* peptide comprises:

TAT	AAA	CTT	GTG	GTA	GTT	GGA
Tyr	Lys	Leu	Val	Val	Val	Gly
						10

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GCT	TGT	GGC	GTA	GGC	AAG	AGT	(SEQ. ID NO. 8)
Ala	Cys	Gly	Val	Gly	Lys	Ser	(SEQ. ID NO. 13)
				15			

or portion or variant thereof.

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In yet another embodiment, the DNA sequence encoding the mutant *ras* peptide comprises:

TAT	AAA	CTT	GTG	GTA	GTT	GGA
Tyr	Lys	Leu	Val	Val	Val	Gly
	5					10

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GCT	GTT	GGC	GTA	GGC	AAG	AGT	(SEQ. ID NO. 9)
Ala	Val	Gly	Val	Gly	Lys	Ser	(SEQ. ID NO. 10)
				15			

or portion or variant thereof.

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The present invention encompasses conservative substitutions based on codon degeneracy, provided that the modification results in a functionally equivalent mutant *ras* peptide or a peptide with enhanced immunogenicity. Included are substitutions in codons in positions distinct from the codon encoding the amino acid at position 12. For example, the codon which encodes the amino acid lysine at position 5 may be replaced by the codon encoding tyrosine.

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The invention further provides vectors and plasmids comprising a DNA sequence encoding a mutant *ras* peptide. The vectors include but are not limited to *E. coli* plasmid, a Listeria vector and recombinant viral vector. Recombinant viral vectors including but not limited to orthopox virus, avipox virus, capripox virus, suipox virus, vaccinia, baculovirus, human adenovirus, SV40, bovine papilloma virus, and the like comprising the DNA sequence encoding a mutant *ras* peptide.

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Recombinant mutant *ras* peptide can be obtained using a baculovirus expression system in accordance with the method of Bei et al J. Clin. Lab. Anal. 9:261-268 (1995). Recombinant viral vectors can be constructed by methods

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known in the art such as U.S. Patent No. 5,093,258; Cepko et al Cell 37:1053-1062 (1984); Morin et al Proc. Natl. Acad. Sci USA 84:4626-4630 (1987); Lowe et al Proc. Natl. Acad. Sci USA 84:3896-3900 (1987); Panicali & Paoletti, Proc. Natl. Acad. Sci USA 79:4927-4931 (1982); Mackett et al, Proc. Natl. Acad. Sci USA 79:7415-7419 (1982); WO 91/19803; Perkus et al Science 229:981-984 (1985); Kaufman et al Int. J. Cancer 48:900-907 (1991); Moss Science 252:1662 (1991); Smith and Moss BioTechniques Nov/Dec, p. 306-312 (1984); U.S. Patent No. 4,738,846; Sutter and Moss Proc. Natl. Acad. Sci USA 89:10847-10851 (1992); Sutter et al Virology (1994); and Baxby and Paoletti Vaccine 10:8-9 (1992).

Host cells which may express the DNA encoding the mutant *ras* peptide carried by vectors or plasmids are prokaryotic and eukaryotic host cells and include but are not limited to E. coli, Listeria, Bacillus species, COS cells, Vero cells, chick embryo, fibroblasts, tumor cells, antigen presenting cells and the like. When the host cell is an antigen presenting cell, the host cell is an antigen presenting cell, the host cell should additionally express an MHC class I molecule.

The invention has been described in detail including the preferred embodiments thereof. However, it will be appreciated by those skilled in the art, upon consideration of this disclosure, may make modifications and improvements thereon without departing from the spirit and scope of the invention as set forth in the claims.

Reference and patents referred to are incorporated herein by reference.

## EXAMPLES

### Example 1

#### Materials and Methods

Patient selection. Adult cancer patients with histologically confirmed diagnosis of adenocarcinoma of the colon, gastrointestinal tract, lung or pancreas with metastatic disease, were screened as potential candidates for this phase I clinical study. Tumor samples from these patients were examined for point mutations in the *K-ras* gene at codon 12 by polymerase chain reaction (PCR) of paraffin-embedded sections by methods known in the art. In addition to fulfilling

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all other essential inclusion criteria as specified in the FDA-approved NCI clinical protocol, those patients presenting the appropriate *K-ras* mutation were then selected for study entry.

Peptides and immunizations. Mutant *ras* 13-mer peptides used as immunogens were synthesized as clinical grade reagents under GMP conditions (Bachem, Torrance, CA: >97% purity by HPLC). Control and additional experimental peptides, which were used *in vitro*, either were prepared commercially (Bachem) or synthesized in the laboratory on an Applied Biosystems Model 432A personal peptide synthesizer (Foster City, CA) using Fmoc chemistry (>90% purity by HPLC). These reagent-grade products were dissolved in aqueous solution at 2 mg/ml, filter-sterilized and stored in aliquots at -70°C. The normal sequence of *ras* p21 reflecting positions 5-17 is Lys-Leu-Val-Val-Val-Gly-Ala-Gly-Gly-Val-Gly-Lys-Ser (KLVVVGAGGVGKS) (SEQ. ID NO. 1). The mutant *ras* 13-mer peptides used as immunogens in this study reflected the substitution of Gly at position 12 with either an Asp, Cys or Val residue. Patients were injected subcutaneously (s.c.) at multiple sites (i.e. deltoid, thigh and abdomen) with the appropriate mutant *ras* 13-mer peptide corresponding to the specific point mutation previously identified in an autochthonous tumor sample. Prior to each vaccination, the peptide was prepared freshly from a lyophilized stock, reconstituted in sterile water, and admixed by vortexing with clinical grade Detox™ adjuvant (RIBI ImmunoChem Research, Hamilton, MT) as described by the manufacturer. The Detox™ adjuvant, supplied in a lyophilized form, was composed of two active immunostimulants: cell wall skeleton (CWS) from Mycobacterium phlei and Monophosphoryl Lipid A (MPL) from Salmonella minnesota R595, and prepared as an oil-in-water emulsion with squalene and Tween 80. The final concentrations of CWS and MPL per vaccination were 250 µg and 25 µg, respectively. A dose-escalation, phase I clinical trial was designed for up to twelve patients divided among four cohorts. Cohorts I, II, III and IV were designated to receive a total of 0.1 mg, 0.5 mg, 1.0 mg and 1.5 mg of peptide per vaccination, respectively, with up to three vaccinations separated one month apart.

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° Isolation and preparation of peripheral blood mononuclear cells (PBMC).

PBMC were isolated from 50 ml of heparinized whole blood by Ficoll-Hypaque density gradient centrifugation (Lymphocyte Separation Medium; Organon-Teknika, Durham, NC) prior to the first vaccination and four weeks after each vaccination.

5 After isolation at each interval, PBMC were cryopreserved under liquid nitrogen in a sterile cocktail of 90% heat-inactivated, pooled human AB serum (Valley Biomedical, Winchester, VA) and 10% dimethyl sulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO). Basic cellular immune responses to mitogens and  
10 antigens were first determined using freshly-thawed, unfractionated PBMC, derived pre-vaccination and post-third vaccination. For all functional assays and cell culture preparation, PBMC were suspended in a RPMI-1640 based-medium, supplemented with 15 mM HEPES buffer (pH 7.4), 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 50  $\mu$ M  $\beta$ -mercaptoethanol, 50  
15  $\mu$ g/ml gentamicin (all from GIBCO/BRL, Gaithersburg, MD) and 10% heat-inactivated human AB serum (Valley Biomedical). Cell surface phenotype of resting and activated lymphocyte cultures was analyzed by direct immunofluorescence. Cells were treated with the appropriate primary monoclonal  
20 antibody (MAb) conjugated to fluorescein isothiocyanate (FITC) (from Pharmingen, San Diego, CA or Becton Dickinson, Mountain View, CA), fixed with 1% paraformaldehyde and evaluated by flow cytometry using a FACScan (Becton Dickinson) for the percentage of positive cells and mean fluorescent  
25 intensity (MFI).

25 *In vitro* stimulation (IVS) cycles for evaluation of CD4<sup>+</sup> T cell responses.

IVS cycles were established to: (i) amplify detection of a low proliferative response, (ii) compare "pre-immune" to "post-immune" lymphocytes over a period to time for assessment of potential peptide-specific reactivity, and (iii) derive  
30 peptide-specific CD4<sup>+</sup> T cell lines in culture for detailed phenotypic and functional characterization. For the first IVS cycle, freshly-thawed PBMC ( $5 \times 10^5$ /well), derived pre-vaccination and post-third vaccination, were incubated in parallel cultures in 24-well plates (Costar, Cambridge, MA) with irradiated (2,000 rads)  
35 autologous PBMC ( $3-5 \times 10^6$ /well) as antigen presenting cells (APC) plus the

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immunizing *ras* peptide (50  $\mu$ g/ml) and recombinant human IL-2 (10 U/ml, Cetus, Emeryville, CA). After incubation for 7 days, viable cells were recovered and retested for peptide-specific reactivity by proliferation assays (see below) and/or re-cultured by continuous weekly stimulation with peptide and IL-2. For subsequent IVS cycles, recovered lymphocytes were maintained at  $2-5 \times 10^5$ /well and APC at  $5 \times 10^6$ /well. After the third or fourth IVS cycle, autologous PBMC were replaced with Epstein-Barr Virus (EBV)-transformed autologous B cells as APC (i.e. B-lymphoblastoid cell line (BLCL) at  $5 \times 10^5$ /well and irradiated at 20,000 rads). Although IL-2 was maintained at 10 U/ml throughout all IVS cycles, the peptide dose was gradually reduced to 5  $\mu$ g/ml for propagation of only Ag-specific T cell cultures.

IVS cycles for evaluation of CD8<sup>+</sup> T cell responses from HLA-A2<sup>+</sup> Patients. Freshly-thawed PBMC (from *ras* patients 32 and 33), derived pre-vaccination and post-third vaccination, were enriched in T lymphocytes by passage over nylon wool columns (Robbins Scientific, Sunnyvale, CA) and then depleted of CD4<sup>+</sup> T cells by negative selection via panning on anti-CD4 MAb-coated T25 flasks (Applied Immune Sciences, Santa Clara, CA). For the first IVS cycle, CD8<sup>+</sup> enriched lymphocytes ( $5 \times 10^5$ /well) were cultured in 24-well plates with irradiated, autologous PBMC ( $5 \times 10^6$ /well) as APC which had been preincubated for 3 hours with the appropriate mutant *ras* peptide (50  $\mu$ l/ml) plus human  $\beta_2$ -microglobulin (10  $\mu$ g/ml) (Calbiochem, San Diego, CA). The mutant *ras* peptide used here [i.e. *ras*5-14(Asp12)], however, represented a shorter sequence of the original immunogen, which was shown to bind to HLA-A2 (see bioassay below). IL-2 (10 U/ml) was added 3 days later. After an additional 7 days, viable cells ( $2-5 \times 10^5$ /well) were re-cultured by weekly stimulation with APC pre-pulsed with peptide/ $\beta_2$ -microglobulin, and IL-2 added one day later. After the third or fourth IVS cycle, autologous PBMC were replaced with EBV-transformed, autologous BLCL ( $5 \times 10^5$ /well) as APC. The peptide dose was gradually reduced to 5  $\mu$ g/ml for propagation of only Ag-specific CTL cultures. CTL activity was evaluated from cultures 5 to 6 days after Ag restimulation.



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° Proliferation response. Freshly-thawed PBMC ( $1.5 \times 10^5$  cells/well) were incubated in 96-well, flat-bottomed plates (Costar) in the absence and presence of mitogens for up to 3 days and antigens for up to 6 days. The mitogens included Phytohemagglutinin-P (PHA-P) and Pokeweed mitogen (PWM) (both from Sigma), while the antigens included tetanus toxoid (kindly provided by Wyeth Laboratories, Marietta, PA) and the indicated *ras* 13-mer peptides. Furthermore, at the end of an IVS cycle, lymphocytes cultures ( $5 \times 10^4$ /well) were examined for peptide-specific proliferation using irradiated APC (i.e. PBMC,  $5 \times 10^5$ /well or BLCL,  $5 \times 10^4$ /well) incubated with varying concentrations of mutant and control *ras* peptides for up to 3 or 4 days. In all assays, cultures were pulsed with [ $^3$ H]-thymidine ( $1\mu$ Ci/well; Amersham, Arlington Heights, IL) for the final 18-24 hour of their incubation. Cells were collected with a TOMTEC MACH II 96 harvester (Wallace, Inc., Gaithersburg, MD) and incorporated radioactivity was measured by liquid scintillation spectroscopy (1205 Betaplate flat bed LS counter; Wallace). MHC class I or II restriction was analyzed in MAb blocking experiments using anti-HLA-A, B, C (clone G46-2.6) or anti-HLA-DR, anti-HLA-DP or anti-HLA-DQ (clones TU36, HI43 or TU169, respectively) (all from Pharmingen). Anti-CD4 (clone OKT4) and anti CD8 (clone OKT8) (both from ATCC) were used in functional assays to confirm the phenotype of the responding T cell subset. Results were expressed as the mean cpm  $\pm$  SEM of triplicate cultures or stimulation index, which was calculated by dividing the cpm of experimental cultures (means counts of triplicate wells) by the cpm of unstimulated control cultures (mean counts of triplicate wells).

HLA-A2 binding bioassay. The 174CEM.T2 cell line ("T2"), (transport deletion mutant) as described in Anderson et al, 1993, *J. Immunol.* 151:3407-3419) was used as a bioassay to measure potential functional binding of exogenously-supplied peptides to human HLA-A2, similarly as described (16) with some modifications. Briefly, T2 cells ( $1 \times 10^6$ /treatment) were incubated in a 24-well plate overnight in serum-free IMDM medium in the absence and presence of the different mutant *ras* peptides ( $50 \mu$ g/ml) plus human  $\beta_2$ -microglobulin ( $10 \mu$ g/ml for Calbiochem or  $3 \mu$ g/ml of purified protein from Intergen, Purchase, NY). The

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CEA<sub>571-579</sub> peptide was included here as a positive control for binding to HLA-A2(17). After incubation, T2 cells were washed free (of unbound peptide and  $\beta_2$ -microglobulin) and stained by indirect immunofluorescence for the phenotypic expression and potential upregulation of HLA-A2 using a HLA-A2-specific MAb (One Lambda, Canoga Park, CA). Additionally, T2 cells were stained with a pan HLA class I MAb, W6/32 and an isotype-matched (IgG<sub>2a</sub>) MAb, UPC-10 (Cappel, West Chester, PA). Data were expressed as the MFI of the gated positive cells.

Cytotoxic response. Cytotoxicity was examined by a conventional <sup>51</sup>Cr-release assay, as described (6). Briefly, target cells (i.e. T2, C1R-A2, BLCL, K562) were radiolabeled with 200  $\mu$ Ci of Na<sub>2</sub>[<sup>51</sup>Cr]O<sub>4</sub> (Amersham) for 60 minutes at 37°C, followed by thorough washing to remove unincorporated isotope. Effectors and targets (1 x 10<sup>4</sup>/well) were incubated in 96-well, U-bottomed plates (Costar) at various effector target (E/T) ratios in the absence and presence of peptide (+/- monoclonal antibody (MAb) directed against CD4, CD8 or HLA-A2 molecules (clone BB7.2 from ATCC)) for up to 6 hours. After incubation, supernatants were collected using a Supernatant Collection System (Skatron, Sterling, VA) and radioactivity measured in a  $\gamma$ -counter (Packard Instruments, Downers Grove, IL). Cytotoxic activity was defined as percent specific release of <sup>51</sup>Cr and determined by the equation: [(experimental<sub>cpm</sub> - spontaneous<sub>cpm</sub>)/maximum<sub>cpm</sub> - spontaneous<sub>cpm</sub>] x 100. Results were expressed as the mean  $\pm$  SEM of triplicate cultures. The C1R-A2 line is a BLCL transfected with the HLA-A2 gene (obtained from Dr. P. Creswell, Yale University) (Storkus et al, 1987 *J. Immunol.* 138:1657-1659), and K562 is an erythroleukemia cell line (ATCC, Rockville, MD) used to measure NK activity.

Immunoassays for detection of mutant K-ras protein in human tumor cell lines.

Proliferation response. Normal and mutant *ras* proteins were derived from various tumor cell lines (ATCC) which included: Calu-1, a lung carcinoma with a Cys12 mutation; SW480, a colon carcinoma with a Val12 mutation; and HT-29, a colon carcinoma with no known *ras* codon 12 point mutation (1,18,19). Detergent-lysates were produced aseptically from each cell line by extraction (at 10<sup>7</sup> cells/ml)

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in buffer containing: 10 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA; 1% NP-40; and protease inhibitors (2  $\mu$ g/ml aprotinin; 0.2 mM PMSF; 0.5  $\mu$ g/ml leupeptin). After extraction in non-ionic detergent and removal of nuclei and debris, protein concentrations in lysates were determined by the BCA Protein Assay Reagent (Pierce, Rockford, IL) and aliquots were stored at -70°C until analysis. In order to specifically separate and capture *ras* proteins from these cellular extracts, they were incubated on wells (of 96-well, flat-bottomed plates; Costar) pre-coated with a pan-*ras* MAb, clone RAS 10 (Oncogene Science, Cambridge, MA). Clone RAS 10 previously has been used as a capture MAb in a sandwich ELISA (20). Briefly, plates were coated with RAS 10 (3  $\mu$ g/ml in 0.05 ml/well or 0.15  $\mu$ g/well) suspended in 0.1 M carbonate buffer (pH 9.6) overnight at 4°C. Wells were then washed with PBS and blocked with PBS containing 5% BSA for 60 minutes at 37°C. Lysates were added at specific protein concentrations and incubated overnight at 4°C with additional BSA (0.5%) and extraction buffer to achieve a final volume of 0.1 ml. Control wells received diluent only (i.e. extraction buffer). Plates were washed thoroughly (6 to 8 times) with culture medium in preparation for the proliferation assay.

*Flow cytometry.* Each cell line was examined for the expression of intracellular *ras* p21 proteins, independent of mutation, by flow cytometry also using the pan-*ras* MAb, clone RAS 10 (Oncogene Science protocols). Cells were washed to remove serum, fixed with 2% paraformaldehyde for 10 minutes at room temperature (at  $10^6$  cells/ml), washed and maintained in PBS buffer containing 10% heat-inactivated goat serum (GIBCO/BRL) plus 0.1% saponin (Calbiochem). Afterwards, cells were treated with the appropriate primary MAb, followed by secondary staining with FITC-conjugated affinity-purified, goat anti-mouse IgG (Southern Biotechnology, Birmingham, AL) and analyzed.

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**Table 1**

*ras p21 Expression in Carcinoma Cell Lines  
As Measured By Flow Cytometry*

	<u>Cell Line <i>a</i></u>		<u>Percent Positive Cells (MFI) <i>b</i></u>			
		<u>isotype</u>	<u>anti-class I</u>	<u>anti-ras(<math>\mu</math>g/ml)</u>		
5				<u>10</u>	<u>3</u>	<u>1</u>
	Calu-1	1.0	93.4 (1617.0)	90.6 (331.8)	87.8 (224.8)	59.8 (176.4)
10	SW480	5.3	84.2 (1184.6)	83.4 (630.9)	82.8 (386.0)	67.0 (273.4)
	HT29	11.6	93.5 (1313.7)	93.1 (422.8)	83.4 (303.9)	58.6 (232.9)
15	<i>a</i>	See Table 4.				
	<i>b</i>	Each cell line was examined for the expression of intracellular <i>ras</i> p21 protein (independent of mutation) by flow cytometry using the pan- <i>ras</i> MAb, clone RAS 10, at different concentrations. W6/32 was included as a positive control for anti-MHC class I staining, while UPC-10 was included as an isotype-matched (IgG2a) control. Results are expressed as percent positive cells with mean fluorescence intensity (MFI) shown in parentheses.				
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*Enzyme Immunoassay.* Similarly, each cell line was also examined for the expression of intracellular *ras* p21 proteins, independent of mutation, by an ELISA. As described above, plates were coated with RAS 10 (0.15  $\mu$ g/well) suspended in 0.1 M carbonate buffer (pH 9.6) overnight at 4°C. At the same time, lysates (300  $\mu$ g of protein) or diluent control were incubated in separate polypropylene tubes with MAb clone Y13-238 (1  $\mu$ g/ml) (Oncogene Science), which is reactive with both K-*ras* and H-*ras* proteins (21) or an isotype-matched MAb (rat IgG<sub>2a</sub> anti-mouse B7.1; Pharmingen). The next day, wells were washed with PBS and blocked with PBS containing 5% BSA for 60 minutes at 37°C. The diluent control or lysates, at specified protein concentrations were added and incubated overnight at 4°C with additional BSA (0.5%) and extraction buffer to

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° achieve a final volume of 0.05 ml. Plates were washed 6 times with PBS containing 0.05% Tween 20 (PBST), and incubated for 60 minutes at room temperature with an affinity-purified, goat anti-rat IgG conjugated to horseradish peroxidase (1:1,000 dilution; Southern Biotechnology). Plates were washed 6  
5 times with PBST and the reactions were visualized following the addition of the chromogen *o*-phenylenediamine dihydrochloride (Sigma) and hydrogen peroxide and measured at an absorbance of 490 nm using an ELISA microplate autoreader (Bio-Tek Instruments, Winooski, VT).

**Table 2**

*ras p21 Expression in Carcinoma Cell Lines as Measured  
by Enzyme Immunoassay*

	<u>Cell Line</u> <i>a</i>	<u>Immunoreactivity</u> ( $A_{490}$ ) <i>b</i>	
		<u>isotype</u>	<u>anti-ras</u> ( $\mu$ g protein)
			<u>75</u> <u>7.5</u>
15	Calu-1	0.299	1.496 1.288
	SW480	0.333	1.587 1.496
	HT29	0.283	1.487 0.990
20	None <i>c</i>	0.288	0.306 0.345
	<i>a</i> See Table 4.		
	<i>b</i> Detergent lysates were isolated from each cell line, and examined for the presence of intracellular <i>ras</i> p21 proteins (independent of mutation) by a sandwich ELISA. To determine anti- <i>ras</i> reactivity, cellular extracts were normalized to the same protein concentrations, as shown. The pan- <i>ras</i> MAb, clone RAS 10 (see Table 2) was used for "capture", while the <i>ras</i> MAb, clone Y13-238, which is reactive with both K- <i>ras</i> and H- <i>ras</i> proteins, was used for "detection". Rat anti-mouse B7.1 was included as an isotype-matched (rat IgG2a) control. Results are reported at $A_{490}$ as the mean of triplicate wells.		
25			
30	<i>c</i> Control wells contained diluent (i.e., extraction buffer) in lieu of lysates.		

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**Example 2****Vaccination of Cancer Patients With Mutant *Ras* Peptides and Production of anti-Mutant *ras*-Specific, MHC Class II-Restricted CD4<sup>+</sup> T-Cell Lines**

A peptide-based, phase I clinical trial was initiated in metastatic carcinoma patients whose tumors harbor point mutations in the *ras* p21 proto-oncogenes at codon 12. Selected patients were vaccinated with a mutated *ras* 13-mer peptide spanning positions 5-17, which corresponded to the specific point mutation at codon 12 found in their cancer. After completion of two of the four cohorts (three patients/cohort), two patients demonstrated peptide-specific cellular immune responses resulting from the vaccination.

**Patient 43; Mutant *ras*5-17(Cys12) Peptide**

Patient 43 received three complete vaccination cycles of mutant *ras*5-17(Cys12) peptide, 0.5 mg/vaccination (i.e., cohort II), given in Detox™ adjuvant. Lymphocytes were propagated *in vitro* by IVS (see Example 1) from post-third vaccinated lymphocytes of RAS patient 43. Antigen-specific, major histocompatibility complex (MHC) class II-restricted CD4<sup>+</sup> T-cell lines were established when cultured on the immunizing peptide [i.e., *ras*5-17(Cys12)] (Figure 1A and 1B). Lymphocyte cultures were also derived pre-vaccination from this patient. The lymphocyte cultures were analyzed and compared for peptide-specific cell-mediated immunity (Table 3). Lymphocyte cultures obtained post-vaccination demonstrated a dose-dependent proliferative response following stimulation with the immunizing peptide, as expressed by both cpm and SI values. In contrast, no proliferative response was detectable following incubation with the normal *ras*5-17(Gly12) peptide, revealing a lack of cross-reactivity with wild-type *ras* and affirming specificity for recognition of the mutated *ras* sequence. Moreover, lymphocyte cultures obtained pre-vaccination failed to proliferate in response to stimulation with the mutant *ras*5-17(Cys12) peptide, even after four IVS cycles on that same peptide (Table 3). While lymphocyte cultures derived post-vaccination continued to proliferate as an Ag-specific cell line *in vitro* (see Figs. 1A and 1B, for example), lymphocyte cultures derived pre-vaccination began to show a substantial reduction in their capacity to grow by IVS cycle 5. Thus, these results

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° suggest that the proliferative activity expressed by the post-vaccine lymphocyte culture likely resulted from *in vivo* priming.

The kinetics for the development of this lymphocyte response was examined and illustrated from IVS cycles 3-7, for example (Fig. 1A). Peptide-specific lymphocyte reactivity increased over time, as measured at two different *ras5-17*(Cys12) peptide concentrations. These data demonstrated that the strength of the peptide-induced proliferative signal increased by IVS cycle, with no detectable cross-reactivity induced or observed against the wild-type *ras* sequence. Also, no detectable proliferation was observed using an irrelevant mutant *ras5-17* peptide which contained Val in place of Cys at position 12 (Fig. 1B), further demonstrating lymphocyte-specific recognition of the mutant *ras5-17*(Cys12) peptide.

Although flow cytometry revealed the phenotype of this lymphocyte culture to be predominantly CD4<sup>+</sup>, anti-CD4-MABs were included in proliferation assays to identify and confirm the functional T cell subset (Fig. 2B). Indeed, anti-CD4 MAB, but not anti-CD8 MAB, inhibited lymphoproliferation, indicating that the peptide-specific proliferative response was mediated by CD4<sup>+</sup> T cells. Additionally, MAB directed against nonpolymorphic determinants of HLA class I and II (DP, DR, DQ) molecules were used to define the nature and requirement of MHC restriction for peptide presentation. The proliferation response was determined to be HLA class II-restricted, which mapped to the HLA-DP allele(s), since MAB directed against HLA-DP, but not HLA-DR, HLA-DQ or HLA class I, effectively inhibited peptide-specific stimulation. Thus, vaccination of patient 43 with mutant *ras5-17*(Cys12) peptide appeared to lead to the *in vivo* priming of an Ag-specific, HLA-DP-restricted CD4<sup>+</sup> T cell response, which was determined *in vitro* by the production and expansion of the precursor population. Although no specific cytotoxic response was detected against peptide-pulsed autologous EBV-B cells as targets, specific IFN- $\gamma$  production was detected in response Ag stimulation.

#### Patient 29; Mutant *ras5-17*(Val12) Peptide

Patient 29 had a duodenum carcinoma as the primary cancer which harbored a K-*ras* mutation at codon 12, encoding the substitution of Gly to Val. Patient 29

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received three complete vaccination cycles of mutant *ras*5-17(Val12) peptide, 1 mg/vaccination (i.e., cohort III), given in Detox™ adjuvant. Patient 29 shared an HLA-A2 allele and patient 29's PBMCs were assessed for evidence of both CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses.

As with patient 43 (Table 3), PBMC from patient 29 obtained post-vaccination and following IVS proliferated in response to stimulation with the immunizing peptide (Fig. 8A). In contrast, no proliferative response was detectable following incubation with the normal *ras*5-17(Gly12) peptide, thus supporting TCR specificity for recognition of the mutated *ras* sequence. PBMC cultures obtained pre-vaccination, when assayed in parallel after the same IVS cycle, failed to proliferate in response to stimulation with the mutant *ras*5-17(Val12) peptide (Fig. 5B). Thus, the proliferative activity expressed by the post-vaccine lymphocyte culture was shown to be likely to result from *in vivo* sensitization. Also, as with patient 43, an Ag-specific T cell line from postvaccine PBMC of patient 29 was propagated and maintained by IVS with autologous EBV-B cells, antigenic peptide, and IL-2. The peptide-specific proliferative response was demonstrated to be mediated by CD4<sup>+</sup> T cells, as anti-CD4 MAb, but not anti-CD8 MAb, inhibited lymphoproliferation (Fig. 8B). Additionally, the proliferative response was shown to be HLA class II-restricted, which mapped predominantly to the HLA-DQ allele, as MAb directed against HLA-DQ, but not HLA-DR, HLA-DP or HLA class L ablated peptide-specific stimulation. Thus, vaccination of patient 29 with mutant *ras*5-17(Val12) peptide appeared to result in the *in vivo* priming of an Ag-specific, HLA-DQ-restricted CD4<sup>+</sup> T cell response. As with patient 43, the CD4<sup>+</sup> T cell line of patient 29 did not display specific cytotoxicity against peptide-pulsed autologous EBV-B cells as targets, although it did produce IFN-gamma, as well as IL-4, in response to specific Ag stimulation.

### Example 3

#### **Ability of the Peptide-derived CD4<sup>+</sup> T-Cell Line of Patient 43 to Recognize the Appropriate Mutant *Ras* Protein**

An important immunologic objective was to determine whether the *ras* peptide-induced T cell lines could also recognize a processed form of the



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corresponding mutant *ras* protein, which may be expressed and presented by either the tumor or the APC population. A model system was developed to examine the capacity of the anti-*ras*5-17(Cys12) peptide-specific CD4<sup>+</sup> T cell line of patient 43 to recognize the mutant *ras* Cys12 protein processed and presented by an autologous APC population (i.e., EBV-B cells) (Table 4). Since a purified or recombinant source of mutant *ras*Cys12 protein was unavailable, the protein was isolated by extraction (with non-ionic detergent) from a human tumor cell, the Calu-1 lung carcinoma, which harbors that particular codon 12 mutation. The HT-29 and SW480 colon carcinoma cell lines, harboring either no known codon 12 mutation or a Val12 mutation, respectively, were used as sources of negative control proteins for monitoring the specificity of the immune reaction. In order to separate and specifically capture total *ras* proteins from these crude detergent - lysates for biologic presentation by an APC population, they were first incubated on wells (of a 96-well plate) pre-coated with a pan-*ras* MAb, clone RAS 10, previously characterized as a "capture" MAb in an ELISA format (20). Unbound material was removed from these wells by extensive washing in physiologic buffer and culture medium prior to adding cells.

Using this model system, it was found that the anti-*ras*Cys12 peptide-specific CD4<sup>+</sup> T cell line, in the presence of EBV-B cells as APC, proliferated in response to the Calu-1 lysate (i.e., "extract") in a dose-dependent fashion (Table 4). The absence of specific CD4<sup>+</sup> proliferation using SW480 or HT-29 lysates strengthened the notion for specific TCR recognition of an epitope(s) expressed by the Calu-1-derived *ras* proteins. Additional control cultures showed productive CD4<sup>+</sup> T cell proliferation in the presence of the specific mutant *ras*5-17(Cys12) peptide in a dose-dependent fashion and the lack of specific proliferation in the absence of peptide or in the presence of the nonmutated *ras* peptide sequence (Table 4). The presence of immobilized pan-*ras* MAb neither stimulated nor inhibited peptide-induced proliferation.

The combination of both EBV-B cells as APC and immobilized pan-*ras* MAb was found to be essential in this model system for stimulating CD4<sup>+</sup> T cell proliferation to the mutant *ras*Cys12 protein (Table 5). In the absence of EBV-B

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cells, but in the presence of immobilized pan-*ras* MAb, no proliferation was observed, indicating that an APC population was required for processing/presentation of the mutant *ras* protein (Table 5). It was likely that Ag processing events were required by the APC population, since pretreatment of EBV-B cells with chloroquine blocked protein- but not peptide induced proliferation. Conversely, in the absence of immobilized pan-*ras* MAb or in the presence of an isotype-matched MAb, despite the presence of EBV-B cells, no specific proliferation was observed (Table 5), suggesting that the anti-*ras* MAb was critical for the specific capture of the Calu-1-derived *ras* proteins. The inability of SW480 and HT-29-derived lysates to stimulate CD4<sup>+</sup> proliferation was unlikely due to the absence or weak expression of the RAS 10 MAb-reactive epitope requisite for efficient binding of *ras* proteins to such substrate (Table 2).

An enzyme immunoassay was developed, which allowed specific detection of *ras* proteins captured by the pan-*ras* MAb (clone RAS 10), independent of the *ras* mutation intrinsic to a tumor cell line. Using this assay format, demonstrable and comparable levels of binding reactivity were found among the three different tumor cell lines (Table 2), indicating that CD4<sup>+</sup> proliferation reflected TCR recognition of a mutant *ras* epitope uniquely expressed by the Calu-1, but not SW480 or HT-29 lysates (Table 4). Comparable intracellular levels of RAS 10 expression were also observed by flow cytometry by analysis of both percent positive cells and MFI of permeabilized, fixed cells.

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**Table 3**

*Effect of Peptide Vaccination on Proliferative Response  
of RAS Patient 43*

5	<u>Culture<sup>a</sup></u>	Peptide in assay <sup>b</sup>	Conc. ( <u>ug/ml</u> )	<u>Proliferation Response<sup>c</sup></u>	
				<u>cpm(±SEM)</u>	<u>SI</u>
5	Pre-Immune	Cys12	100	736±320	0.8
			30	1,015±48	1.1
			10	1,076±8	1.2
			3	875±64	1.0
10		Gly12	100	790±456	0.9
			30	849±14	1.0
			10	768±257	1.1
			3	824±255	0.9
15		None (T cell+APC)	--	888±31	--
20	Post-Immune	Cys12	100	14,711 ± 1322	22.0
			30	7,334 ± 294	10.9
			10	5,730 ± 946	8.5
			3	5,935 ± 272	8.9
25		Gly12	100	640±23	1.0
			30	819±142	1.2
			10	725±27	1.1
			3	694±19	1.0
30		None (T cell+APC)	--	670±6	--

*a* Unfractionated PMBC, pre-vaccination and post-third vaccination. Cultures were derived by continuous weekly IVS using autologous PBMC as APC incubated with mutant *ras* 13-mer peptide and IL-2. PBMC cultures shown were following **fourth IVS**.

*b* Mutant, *ras*5-17(Cys12); normal, *ras*5-17(Gly12).

*c* Proliferation measured by <sup>3</sup>H-thymidine uptake. Results expressed as cpm (± SEM of triplicate wells) and stimulation index (SI).

Further, the response was antigen specific and MHC class II-restricted (Figure 2A and B).

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**Table 4**

*CD4+ T Cell Line of RAS Patient 43 Proliferates in Response to Mutant K-ras Protein<sup>a</sup>*

	Antigen in assay <sup>b</sup>	Conc. <sup>c</sup>	K-ras Mutation <sup>d</sup>	Proliferation Response <sup>e</sup>
5	Cys12 peptide	30 10 3 1	-- -- -- --	<b>112,354 ± 413</b> <b>109,519 ± 4942</b> <b>116,529 ± 4947</b> <b>48,732 ± 473</b>
10	Gly12 peptide	30	--	8,130 ± 314
	None (T cell + APC)	--	--	6,836 ± 307
15	Calu-1 extract	250 75 25	Cys12	<b>64,357 ± 2824</b> <b>55,540 ± 1172</b> <b>6,884 ± 445</b>
	SW480 extract	250 75 25	Val12	6,163 ± 1073 4,963 ± 1075 7,682 ± 248
20	HT-29 extract	250 75 25	None (Gly12)	6,955 ± 384 7,223 ± 179 6,835 ± 119

<sup>a</sup> The CD4<sup>+</sup> T cell line of RAS patient 43 (see Table 3) now maintained in culture using autologous BLCL as APC.

25 <sup>b</sup> Assay wells pre-coated with pan-ras MAb clone RAS 10 (0.15 µg/well) before addition of cellular extracts or extraction buffer. Wells then thoroughly washed before addition of APC (i.e., BLCL), T cells ± peptides. Control experiments revealed no effects of the pan-ras MAb on peptide-induced proliferation. Calu-1, lung carcinoma; SW480 and HT-29, colon carcinomas.

30 <sup>c</sup> Peptide expressed as µg/ml; cellular extract or lysate expressed as µg protein.

<sup>d</sup> Corresponds to K-ras mutation at codon 12 and confirmed by PCR analysis.

<sup>e</sup> Proliferation measured by <sup>3</sup>H-thymidine uptake and results expressed as cpm ± SEM of triplicate wells (cpm of irradiated BLCL = 4,181 ± 330).

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Example 4**Identification of a Nested Peptide Sequence With MHC Class I (HLA-A2)  
Binding Activity**

The induction of CD8<sup>+</sup> T cell reactivity was examined. Such CD8<sup>+</sup> T cell activity could have occurred concomitantly with, or in the absence of, the CD4<sup>+</sup> T cell response. In this regard, *in vivo* priming of the CD8<sup>+</sup> T cell response could result from the *in vivo* processing of the immunogen with the subsequent generation of MHC class I-reactive epitopes. In a murine model, overlapping MHC class II-restricted CD4<sup>+</sup> and MHC class I-restricted CD8<sup>+</sup> CTL peptide epitopes reflecting the *ras* Val12 mutation were identified. To this end, the *ras*5-17 peptide sequences were scanned for putative HLA class I consensus anchor motifs reflecting the HLA-A2 allele, as a working model system. The *ras* 10-mer sequence 5-14, independent of *ras* mutation, was identified as a potential candidate for HLA-A2 binding, since it contained the preferred dominant anchors, Leu and Val, at the second (i.e., Leu6) and C-terminus (i.e., Val14) positions of the peptide, respectively (43,44). The *ras* 9-mer sequence 4-12 appeared to satisfy the motif for binding interactions at positions 1 (i.e., Tyr4) and 9 (i.e., Val12). The N-terminal location of the first dominant anchor, Leu, was found at position 3 (i.e., Leu6).

Accordingly, a panel of *ras*5-14 peptides were synthesized, containing either the mutated or wild-type residues at position 12. Subsequently, the capacity of these *ras* peptides to bind to HLA-A2 was analyzed by the T2 bioassay and flow cytometry for specific up-regulation of that class I molecule (Table 7). CEA<sub>571-579</sub>, a 9 amino acid peptide which is a CTL epitope restricted by HLA-A2, was included in these experiments as a positive control. Incubation of T2 cells with mutant *ras*5-14 peptides having either the Asp12, Cys12 or Val12 substitutions, led to enhanced immunostaining with an anti-HLA-A2-reactive MAb as well as with a "pan" class I MAb (W6/32), compared with the no peptide control, thus suggesting functional binding to the HLA class I A2 allele. Additionally, incubation of T2 cells with the wild-type *ras*5-14(Gly12) peptide resulted in increased anti-HLA-A2

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- ° staining, indicating that neither the absence nor the presence of the mutated residue greatly impacted binding to HLA-A2.

The influence of peptide size and relative location of residues surrounding position 12 on HLA-A2 binding were examined using a spectrum of *ras* peptides (Table 7). The 13-mer *ras*5-17(Asp12) peptide, for example, which contained the core residues for binding to HLA-A2, did not appear to bind to T2 cells under these (serum-free) assay conditions, suggesting a critical peptide length and/or required location of MHC anchor residues. Similar results were observed with *ras*5-17(Val12) and *ras*5-17(Cys12) peptides. Moreover, the specificity of binding of these *ras*5-14 peptides to HLA-A2 on T2 cells was further illustrated using a set of mutant *ras*Val12 peptides that: (i) lacked either one [i.e., *ras*5-13(Val12)] or both [i.e., *ras*8-16(Val12)] dominant anchor residues, despite having an appropriate length; and (ii) lacked a desirable length, despite having dominant anchor residues in the second and C-terminus positions [i.e., *ras*5-12(Val12)].

#### **Example 5**

#### **Production of an Anti-*ras*Asp12-Specific, MHC Class I-Restricted CD8<sup>+</sup> T Cell Line from Patient 32**

The above-described mutant *ras*5-14 peptides were used as *in vitro* immunogens and tested for their ability generate Ag-specific CD8<sup>+</sup> CTL lines from HLA-A2<sup>+</sup> patients, pre- versus post-vaccination to determine whether the *ras*5-14 peptide sequences represented HLA-A2-restricted, CD8<sup>+</sup> CTL epitopes and whether *ras* peptide vaccination led to the induction of CD8<sup>+</sup> T cell responses.

Patient 32, who expressed an HLA-A2 allele, had colon carcinoma as the primary cancer harboring a K-*ras* mutation at codon 12, encoding the substitution of Gly to Asp. Patient 32 received three complete vaccination cycles of mutant *ras*5-17(Asp 12) peptide, 0.1 mg/vaccination (i.e., cohort I), administered in Detox™ adjuvant. No specific CD4<sup>+</sup> proliferative response was observed by post-vaccine PBMC against the immunizing 13-mer *ras* peptide even after multiple IVS cycles; however, this did not preclude *in vivo* processing of the peptide and generation of HLA class I-reactive epitopes for potential *in vivo* priming of CD8<sup>+</sup> T cells.

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CD8<sup>+</sup> T lymphocytes, isolated pre-vaccination and post-third vaccination from RAS patient 32, were propagated by IVS (Example 1), and then were analyzed and compared for the development of peptide-specific, cell-mediated cytotoxicity against C1R-A2 targets (Table 6). CD8<sup>+</sup> lymphocyte cultures obtained post-vaccination began to express peptide-specific, cell mediated lysis starting at IVS cycle 6, with lytic potency increasing over time. No specific cytotoxic response was detectable in the presence of the normal *ras*5-17(Gly12) peptide, revealing a lack of cross-reactivity with wild-type *ras* and affirming specificity for recognition of the mutated *ras* sequence. In contrast, CD8<sup>+</sup> lymphocyte cultures obtained pre-vaccination failed to display specific cytotoxicity. Moreover, while CD8<sup>+</sup> lymphocyte cultures derived post-vaccination continued to proliferate efficiently as an Ag-specific cell line *in vitro* (Table 6), CD8<sup>+</sup> lymphocyte cultures derived pre-vaccination lost their capacity to grow beyond IVS cycle 7. Thus, the cytotoxic activity expressed by the post-vaccine lymphocyte culture of patient 32 likely resulted from *in vivo* priming, and the *ras*5-14(Asp12) peptide sequence reflected a HLA-A2-restricted CD8<sup>+</sup> CTL epitope.

Peptide-specific, cell-mediated cytotoxicity expressed by the T cell line of patient 32 was confirmed to be mediated by CD8<sup>+</sup> T cells, as MAb directed against the CD8, but not the CD4, molecule abolished the lytic response (Fig. 7). Additionally, MAb directed against HLA-A2 (clone BB7.2) on the target cell inhibited cytotoxicity (Fig. 7), thus demonstrating a requirement of HLA-A2 for peptide presentation, which was consistent with the binding characteristics of the peptide (i.e., by the T2 bioassay; Table 7).

The fine specificity for TCR recognition of peptide for lysis was examined using a panel of *ras* peptides (Fig. 5). Of the *ras*5-14 peptides tested, only *ras*5-14(Asp12) sensitized the T2 cell line for lysis. In contrast, *ras*5-14 peptides, reflecting the wild-type sequence (Gly12) or an irrelevant mutation (Val12), failed to stimulate lysis, despite their ability to bind to HLA-A2 (Table 7). Furthermore, *ras*5-17(Asp12), which was used as the original immunogen for vaccination, did not appear to induce cytotoxicity under these assay conditions, suggesting a failure to generate the appropriate peptide fragment *in vitro* and/or to produce a sufficient

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concentration of the relevant epitope to induce a detectable lytic response. The lack of lysis against K562 cells (Fig. 5) argued against the contribution of NK activity in these cytotoxic reactions. Thus, both amino acid sequence and peptide length were shown to be important for HLA-A2 binding and/or TCR recognition requisite for anti-*ras* CD8<sup>+</sup> CTL-mediated lysis.

#### Example 6

#### **Production of an anti-*ras*Val12-Specific, MHC Class I-Restricted CD8<sup>+</sup> T Cell Line from Patient 29**

The generation of an HLA-A2-restricted CD8<sup>+</sup> CTL response was examined in patient 29. As a result of the limited availability of whole blood obtained from this patient (post-third vaccination), a T cell line was generated from unfractionated PBMC without CD8<sup>+</sup> T cell enrichment. In addition, because no CD4<sup>+</sup> T cell response was observed with pre-vaccine lymphocyte cultures from patient 29 (Fig. 8A), suggesting the unlikelihood for a detectable pre-existing immune response, only post-vaccine lymphocytes were examined for CD8<sup>+</sup> T cell reactivity. Also, as described hereinabove, for patients 43 and 32, only the post-vaccine lymphocyte cultures displayed peptide-specific CD4<sup>+</sup> or CD8<sup>+</sup> T cell activity, respectively. In contrast, under the same culture and assay conditions, no detectable pre-existing peptide-specific immune responses were observed in pre-vaccine lymphocytes of those same patients.

Lymphocytes from patient 29 were propagated *in vitro* with the homologous mutant *ras* 10-mer peptide [i.e., *ras*5-14(Val12) herein] as immunogen, which reflected its ability to bind to HLA-A2 (Table 7). As shown, following IVS cycle 7, such lymphocytes displayed peptide-specific, cell-mediated lysis against C1R-A2 targets incubated in the presence of the *ras*5-14(Val12) peptide, but not in the presence of the normal *ras*5-17(Gly12) peptide (Fig. 9A). Peptide-specific, cell-mediated cytotoxicity expressed by the T cell line of patient 29 was shown to be mediated by CD8<sup>+</sup> T cells, as MAb directed against the CD8, but not CD4, molecule inhibited the lytic response (Fig. 9B). Phenotypic analysis of this T cell revealed it to be > 90% CD8<sup>+</sup>, as analyzed by IVS cycle 8. In addition, MAb directed against HLA-A2 (clone BB7.2) on the target cell (i.e., autologous EBV-B



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cells) inhibited cytotoxicity (Fig. 9B), thus demonstrating a requirement for HLA-A2 restriction. In view of the results of this Example and those above, the production of anti-*ras*Val12-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses in the same patient was demonstrated (Figs. 8A, 8B, 9A, 9B).

Studies were also carried out to determine whether such peptide-induced CD8<sup>+</sup> CTL could recognize a processed form of the corresponding mutant *ras* protein. To test this hypothesis, the functional interaction between CD8<sup>+</sup> T cells of patient 29 and the SW480 colon carcinoma cell line was examined. SW480 endogenously expressed both the appropriate MHC restriction element (HLA-A2) and the appropriate *ras* mutation at codon 12 requisite for CTL recognition (18). In contrast to the CD4<sup>+</sup> analysis, which measured lymphoproliferation to soluble protein, the CD8<sup>+</sup> CTL analysis reflected lytic ability against SW480 cells harboring the naturally-occurring mutation in the absence of exogenously added peptide. Furthermore, SW480 cells were tested untreated or following a short term pretreatment with IFN-gamma, which may be responsible for up-regulating the expression of HLA-A2, as well as other events potentially associated with epitope processing and presentation.

Under these assay conditions, IFN-gamma (IFN- $\gamma$ ) pretreatment enhanced the percentage of SW480 cells positive for HLA-A2 and the density of HLA-A2 molecules per cell based on the MFI without inducing detectable alterations in the expression of the *ras*Val12 protein as determined by ELISA. In the absence of IFN- $\gamma$  pretreatment, no CTL lysis was detectable; however, following IFN- $\gamma$  pretreatment, demonstrable cytotoxicity was observed at multiple effector/target ratios (Fig. 10A), which correlated with enhanced HLA-A2 and ICAM-1 expression on SW480 cells. Effector cell specificity of lysis was revealed by the inability of an irrelevant HLA-A2-restricted, CD8<sup>+</sup> CTL line (i.e., anti-MART-1<sub>27-35</sub> peptide-specific) to lyse SW480 cells (with or without IFN- $\gamma$ ) (Fig. 10A), unless the appropriate exogenous peptide was added (Fig. 10B). Anti-MART CTL also failed to lyse SW480 target cells (with or without IFN- $\gamma$ ) in the presence of exogenously added *ras*5-14(Val12) peptide (Fig. 10B), thus demonstrating specificity for recognition of MART-1, but not *ras* epitopes.

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By contrast, anti-*ras*Val12 CTL-mediated lysis of SW480 cells was enhanced in the presence of exogenously added *ras*5-14(Val12) peptide, but not wild-type *ras* peptide, suggesting that the presentation of the endogenously-produced mutant *ras* epitope(s) was limiting. Target cell specificity for lysis was revealed by the inability of anti-*ras*Val12 CTL to lyse HLA-A2+ melanoma cells (with or without IFN- $\gamma$ ), which lacked the *ras*Val12 mutation in the absence of exogenous peptide. The observation that either CTL line lysed IFN- $\gamma$ -pretreated SW480 cells more efficiently than untreated SW480 cells when incubated in the presence of their respective relevant peptide may have been due, in part, to the increased expression of cell surface HLA-A2 available for peptide loading, thereby, creating more antigenic complexes for CTL recognition.

**Table 5**

*Requirements for CD4+ T Cell Response to Mutant K-ras Protein*

Antigen in assay <sup>a</sup>	Conc. <sup>b</sup>	Ab Coating (+/-) <sup>c</sup>	BLCL (+/-) <sup>e</sup>	Proliferation Response <sup>f</sup>
Cys12 peptide	10	+	+	87,889 $\pm$ 3329
Gly12 peptide	10	+	+	8,844 $\pm$ 753
None (T cell + APC)	-	+	+	8,642 $\pm$ 206
Calu-1 extract	250	+	+	80,534 $\pm$ 2207
		-	+	7,185 $\pm$ 93
		+ <sup>d</sup>	+	7,315 $\pm$ 137
		+	-	335 $\pm$ 42

*a/b* See footnotes "b/c" of Table 4.

*c* Assay wells with (+) or without (-) pre-coating of pan-*ras* MAb clone RAS 10.

*d* Assay wells pre-coated with an isotype-matched MAb (clone UPC-10).

*e* Assay conducted with (+) or without (-) autologous BLCL as APC.

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- <sup>o</sup> *f* Proliferation measured by <sup>3</sup>H-thymidine uptake and results expressed as cpm  $\pm$  SEM of triplicate wells (cpm of irradiated BLCL = 8,520  $\pm$  225; cpm of T cells alone without BLCL = 339  $\pm$  49).

**Table 6**

*Comparison of Cytolytic Activity by CD8+ Cultures of RAS  
Patient 32 Pre-Immunization and Post-Immunization*

	<u>Culture</u> <sup>a</sup>	<u>IVS Cycle</u> <sup>b</sup>	<u>Recovery</u> <sup>c</sup> (fold increase)	<u>% Specific Lysis</u> <sup>d</sup>		
				<u>Mutant</u>	<u>Control</u>	<u>None</u>
10	Pre- Immune	6	2.3	14 $\pm$ 0	10 $\pm$ 1	13 $\pm$ 1
		7	1.3	0	0	0
		8	0.2	NA <sup>e</sup>	NA	NA
15	Post- Immune	6	6.5	24 $\pm$ 2	8 $\pm$ 0	13 $\pm$ 1
		7	3.5	26 $\pm$ 2	8 $\pm$ 1	2 $\pm$ 0
		8	7.2	67 $\pm$ 2	10 $\pm$ 1	8 $\pm$ 0
		9	2.5	68 $\pm$ 4	21 $\pm$ 2	22 $\pm$ 0

- <sup>a</sup> In a second independent experiment, CD8<sup>+</sup> T cells were isolated from PBMC, pre-vaccination and after the third vaccination.

<sup>b</sup> Cultures were initiated and maintained by continuous weekly IVS using autologous PBMC as APC (up to cycle 3) or BLCL (thereafter) incubated with mutant *ras* peptide plus  $\beta_2$ -microglobulin and IL-2. The mutant *ras* peptide represented a nested HLA-A2 binding, 10-mer sequence [(i.e., *ras*5-14(D12))].

- <sup>c</sup> Cell growth as depicted by the ratio of cells recovered pre and post each IVS cycle.

<sup>d</sup> Cytotoxicity was determined by a standard 6 hr <sup>51</sup>Cr-release assay using the C1R-A2 cell line as a target, incubated with and without *ras* peptides (i.e., 10-mer sequences @ 10  $\mu$ g/ml), as shown. Results illustrated at E/T ratio of 20/1.

<sup>e</sup> NA, no cells available for assay due to lack of growth.

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**Table 7**

*Identification of Mutant ras Peptides that Bind to Human HLA-A2*

	<u>Peptide</u> <sup>a</sup>	<u>Monoclonal Antibody Directed Against</u> <sup>b</sup>		
		<u>HLA-A2</u>	<u>Pan Class I</u>	<u>Isotype</u>
5	None	137.9	230.4	84.0
	<i>ras</i> 5-17(D12)	162.8	236.0	46.5
	<i>ras</i> 5-14(D12)	<b>348.3</b>	<b>561.7</b>	50.8
10	<i>ras</i> 5-14(G12)	<b>285.2</b>	<b>522.8</b>	41.3
	<i>ras</i> 5-14(C12)	<b>361.4</b>	<b>650.9</b>	61.4
	<i>ras</i> 5-14(V12)	<b>421.6</b>	<b>652.2</b>	38.1
	<i>ras</i> 5-13(V12)	130.6	214.8	54.1
	<i>ras</i> 5-12(V12)	165.1	265.9	44.2
15	<i>ras</i> 8-16(V12)	146.3	223.3	39.6
	CEA <sub>571-579</sub>	<b>679.5</b>	<b>991.2</b>	48.4

20 *a* *ras* peptides, reflecting the wild-type sequence and three distinct codon 12 mutations; CEA<sub>571-579</sub>, recently reported as a HLA-A2-restricted, CEA-specific CD8<sup>+</sup> CTL epitope, and used here as a positive control peptide for MHC class I binding.

25 *b* The capacity of each peptide to bind to MHC class I (HLA-A2) was determined by a modification in the T2 bioassay. T2 cells were incubated overnight at 37°C with the appropriate peptide (50 µg/ml) + purified human β<sub>2</sub>-microglobulin (3 µg/ml) in serum-free medium. After culture, cells were washed, stained with the appropriate MAb, and specific immunoreactivity evaluated by flow cytometry. Results are expressed as MFI.

30 In summary, the mutant *ras* protein surrounding position 12 was examined for potential HLA-A2 peptide binding motifs. An overlapping or "nested" 10-mer peptide was identified [i.e., *ras*5-14(Asp12)], which was shown to bind to HLA-A2 and display specific functional capacity for expansion of the *in vivo*-primed CD8<sup>+</sup> CTL precursors (Figures 4 and 5). Importantly, no specific CTL responses were

35 detectable against the normal proto-*ras* sequence and no CTL line was produced

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from pre-immune lymphocytes (Table 6). In contrast to the nested 10-mer peptide, the longer 13-mer peptide used as the immunogen did not show detectable binding to HLA-A2 by bioassay (Table 7) and failed to sensitize targets for lysis in vitro (Figure 5). Thus, immunization with *ras*5-17(Asp12) must have been processed in vivo thereby generating the appropriate 10-mer fragment which then led to peptide-specific CD8<sup>+</sup> CTL activation. Although initially identified for the Asp12 substitution, *ras*5-14 peptides reflecting other K-*ras* mutations at codon 12 were also immunogenic and represent HLA-A2-restricted, mutant *ras* CD8<sup>+</sup> CTL epitopes, as the HLA-A2 peptide binding motif in the *ras*5-14 sequence remains unaltered.

#### Example 7

Concomitant experiments in the mouse (BALB/c; H-2<sup>d</sup>) led to the identification of a mutant *ras* 9-mer peptide reflecting the substitution of Gly12 to Val12 [i.e., *ras*4-12(V12)], which resulted in the induction of MHC class I-restricted CD8<sup>+</sup> CTL responses. The basis of immunogenicity, in part, was attributed to the introduction of the mutated Val12 residue, which created a dominant C-terminus anchor for MHC class I binding. The resulting CD8<sup>+</sup> CTL response, thus, likely reflected T cell receptor recognition of a previously unseen peptide/MHC complex. In contrast to the *ras*5-14 sequence, *ras*4-12(V12) only partly shared the consensus motif for HLA-A2 binding, as the leucine anchor shifted from position 2 to 3. Importantly, the introduction of the mutated Val12 residue, as in the mouse, now created a dominant C-terminus anchor. Despite the alteration in the preferred location of the putative leucine anchor, the *ras*4-12(V12) peptide displayed specific, albeit weak, binding to HLA-A2, as detected by bioassay. In contrast, the proto-*ras* sequence, *ras*4-12(G12), failed to bind to HLA-A2, demonstrating specificity of binding of the mutant 9-mer sequence. A CD8<sup>+</sup> CTL line specific for *ras*4-12(V12) was then derived from a normal HLA-A2<sup>+</sup> individual using a model antigen presentation system, i.e., T2 cells pulsed with exogenous *ras*4-12(V12) peptide as APC for the *in vitro* derivation of antigen-specific CTL. (See Figure 6).

To further enhance the immunogenic strength of this APC system, additional B7.1 surface expression (for costimulation) was provided on T2 cells by

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transient infection with a recombinant vaccinia virus (rV-B7.1). In control experiments using an alloreactive system, it was found that under these conditions, B7.1 surface expression on T2 cells increased by 2-3-fold (by MFI), with nominal effect on class I expression, and accelerated the development and/or lytic potency of the resulting allo-HLA-A2-reactive CTL response. This model system was then applied for the generation of a peptide specific CTL response. Indeed, as described herein, a CD8<sup>+</sup> CTL line was produced *in vitro* from a normal HLA-A2<sup>+</sup> donor, which displayed peptide specific and HLA-A2-restricted cytotoxicity against peptide-pulsed targets. No lysis was detectable using the wild-type *ras* peptide to sensitize target cells.

Overall, these findings demonstrate for the first time the definition of mutant K-*ras* HLA-A2-restricted, CD8<sup>+</sup> CTL epitopes at codon 12. Evidence is provided from peripheral blood lymphocytes of both normal individuals and carcinoma patients, which support the hypothesis that vaccination with oncogene-derived peptides induces highly specific and systemic anti-*ras* cellular immune responses. Moreover, the identification and development of these novel 9-mer or 10-mer mutant *ras* peptides has important implications for both active (i.e., vaccination) and passive (i.e., *ex vivo* expansion for cellular adoptive transfer) immunotherapies, which may be used for the induction and propagation of specific CD8<sup>+</sup> CTL responses in cancer patients.

#### Example 8

##### Identification of Mutant *ras* CD8<sup>+</sup> CTL Peptide Variants

As in the mouse model, potential variants of defined peptide epitopes that may increase i) binding to HLA and ii) the capacity to expand T cell precursors *in vitro* without altering TCR specificity can be tested in the HLA-A2 system using the mutant *ras*5-14 peptides reflecting the Asp12 or Val12 substitutions. Although these peptides were identified as HLA-A2-reactive CD8<sup>+</sup> CTL epitopes from vaccinated patients, they appeared to exhibit rather weak binding to HLA-A2 (as described above), and required exogenous  $\beta_2$ -macroglobulin to further improve MHC/peptide interaction. The identification of peptide variants that strengthen or increase the stability or half-life of the MHC/peptide complex (*in vivo* and *in vitro*)

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may enhance the potency of intrinsically weak immunogenic peptides for the induction and amplification of the relevant T cell response. Since the consensus anchor motif for HLA-A2 is known in the art, putative *ras* peptide variants will be synthesized reflecting amino acid substitutions at either primary or secondary anchor positions.

Because the *ras*5-14 sequence already contains the preferred amino acid residues at the dominant anchor positions, 2 (Leu6) and 10 (Val14) of the peptide sequence, they are likely to remain unaltered. Instead, substitutions are introduced at the putative secondary anchor positions, 1 and 3, of the peptide sequence. Candidates include the replacement at position 1 with a Tyr residue or the replacement at position 3 with either a Trp, Leu, Tyr or Phe residue, as well as double substitutions at both positions 1 and 3. Variant peptides reflecting the appropriate *ras* codon 12 mutation will be synthesized containing these substitutions. In comparative studies with the native peptide sequences, these variants are first examined and screened for their ability to bind to HLA-A2 by the T2 bioassay or a functional competition bioassay which measures inhibition of a control HLA-A2-restricted, Ag-specific CTL response. Variants that display enhanced binding to HLA-A2, are then assessed compared with the native peptide sequences, for their capacity to: (a) sensitize targets for lysis using established anti-*ras* CTL lines; (b) stimulate proliferation and expansion of established anti-*ras* CTL lines; and (c) generate anti-*ras* CTL lines from the original source of immune lymphocytes.

Using the anti-*ras*5-14(Asp12) CTL line in accordance with the present invention, it has been found that single-substituted Tyr and Trp variants displayed higher binding activity than did the native peptide sequence to HLA-A2 on T2 cells, with less dependency for exogenous  $\beta_2$ -macroglobulin. In addition, the Tyr variant appears to sensitize targets for lysis better than the native peptide sequence, with a shift in the dose-response by three-fold based on half-maximal activity (Figure 11). The mutant *ras* peptide 5-14(Asp12), SEQ. ID NO. 3, having an N-terminal Tyr residue (e.g., SEQ. ID NO. 11) enhanced cytotoxic T cell activity against antigen-pulsed target cells.

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° The identification of such peptide variants, which increase immunogenicity *in vitro*, promise to be important and effective reagents for subsequent T cell cloning and precursor frequency analysis. Moreover, peptide variants may be used to improve expansion of Ag-specific T cell lines/clones for potential clinical use in adoptive transfer.

#### Example 9

#### CD4<sup>+</sup> and CD8<sup>+</sup> T Cell Subtypes in Cancer

An important aspect of the T cell subset balance is the insight that this balance provides into the immunological basis of disease. Indeed, a host of pathological processes, as determined mainly in model systems of infectious disease, allergy and autoimmunity and, to some extent, cancer have been associated with the balance of the T<sub>H</sub>1/T<sub>H</sub>2 CD4<sup>+</sup> subset response and the nature of the resulting cytokine patterns. Little is known regarding the precise roles of Tc1 and Tc2 CD8<sup>+</sup> subsets in disease, particularly in neoplasia. Although exceptions to the following patterns may exist, in general, type 1 T cell clones have been shown to principally and selectively secrete IL-2, IFN- $\gamma$  and TNF- $\beta$ /lymphotoxin. Type 2 T cell clones have been described to primarily and selectively produce IL-4, IL-5, IL-6, IL-10 and IL-13. Based on the spectrum and functional properties of the different cytokine patterns, type 1 T cells have been implicated in cell-mediated immunity, while type 2 T cells have been proposed to participate in humoral immunity. Accordingly, based on their involvement in cell-mediated immunity, the induction and expression the type 1 T cell response has been proposed as the more relevant and desirable pathway for antitumor reactivity.

Similar to the murine system, in human cancer, potential changes in the development and balance of the "T<sub>H</sub>1/T<sub>H</sub>2" and "Tc1/Tc2" phenotypes can be correlated. To that end, CD4<sup>+</sup> and CD8<sup>+</sup> T cells are purified from patient peripheral blood lymphocytes obtained pre and post-each vaccination cycle, and stimulated *in vitro* under maximal conditions with anti-TCR MAb. Thereafter, cytokine levels, e.g., IFN- $\gamma$  and IL4, IL-5 or IL-10, are determined by ELISA or Elispots assays, as prototype cytokines for type 1 and type 2 responses, respectively. Furthermore, cytokine profiles of CD4<sup>+</sup> and CD8<sup>+</sup> T cell lines



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produced from immunized patients in response to activation with anti-TCR MAb versus antigenic peptide can be evaluated as a more physiological stimulus. The results from these studies may serve as surrogate endpoints in anticancer vaccine clinical trials, and also shed light on potential mechanisms of immune suppression during cancer progression (i.e., bias toward type 2 patterns) and immune enhancement associated with objective tumor responses (i.e., bias toward type 1 patterns). Such correlations may also help in the design of appropriate adoptive transfer culture strategies, such as those described in murine studies, that exploit the identification, selection and amplification of the most relevant T cell subsets.

#### **Example 10**

##### ***ras* Oncogene-Specific T Cells: Isolation and Analysis of Effector Mechanisms**

The ability of epitope-specific T cell populations to recognize processed forms of the endogenously-produced oncoproteins expressed by tumor cells is absolutely crucial to the success of techniques such as ASI or cellular adoptive immunotherapy. In both normal donors and cancer patients, the present invention relates to the hypothesis of processing and presentation of the corresponding peptides (or closely related sequences) as multiple (sub)dominant epitopes with productive TCR recognition of the antigenic complexes. However, in order to elicit a measurable anti-*ras*Val 12 CTL response in the absence of exogenous peptide, for example, the SW480 tumor targets, the cells required a short term prior exposure to IFN- $\gamma$ . Although the exact mechanisms by which IFN- $\gamma$  acted in this model remain to be fully elucidated, cytotoxicity did correlate, at least in part, with increased expression of HLA-A2, ICAM- 1 and Fas (CD95) molecules, suggesting the following nonlimiting and nonbinding theoretical possibilities: (a) the endogenously-produced mutant *ras* epitopes were limiting, but could be enhanced by increasing the density of class I/peptide complexes available for TCR recognition; (b) the avidity of the CTL/target interaction was weak, but could be enhanced by increasing the density of accessory molecules important for Ag-independent interactions; or (c) tumor cell sensitivity to CTL-mediated lysis via a FasL/Fas-dependent apoptotic pathway was originally defective or weak, but could be restored or enhanced by increasing the density of cell surface Fas. These

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possibilities, as well as others, may provide explanations by which tumor cells escape immune recognition and attack, which may occur at the level of TCR-MHC/peptide, cell-cell adhesion or the cytotoxic mechanism. Conversely, the observation that cytotoxicity could be enhanced by IFN- $\gamma$  introduces the possibility for the application of IFN- $\gamma$  or IFN- $\gamma$ -inducing cytokines, such IL-12, in ASI.

The role of a Fas-dependent pathway in anti-*ras*Val12 CTL-mediated lysis of IFN- $\gamma$ -pretreated SW480 tumor cells can be explored in blocking experiments using commercially-available anti-CD95 MAb (clone ZB4), anti-CD95L MAb (clones 4H9 or 4A5) or the combination of both for maximum neutralization. Isotype-matched Ab and Fas-sensitive, autologous peptide-pulsed EBV-B cells will be used as appropriate controls. Additional control experiments include a comparison of untreated to cytokine-pretreated SW480 tumor cells for sensitivity to Fas Ab-induced apoptosis/cell death (using commercially-available clones DX2 or CH-11), as determined by isotope release or TUNEL assays. Jurkat or EBV-B cell lines are used as positive controls. Additional support for the role of Fas in the lytic mechanism involves the use of membrane-permeable peptide-based inhibitors, which have been shown to block apoptotic death in Fas-sensitive cell types, such as Jurkat tumor cells. These inhibitors, when preloaded into susceptible cells, specifically block the functional activity of certain endogenous intracellular cysteine proteases (e.g., caspases, most notably ICE-like and CPP-32-like subfamilies) involved in the biochemical pathway of apoptosis. Taken collectively, these experiments can provide insights into the role of Fas and apoptotic pathways in tumor cell susceptibility to CTL-mediated lysis and modulation of their lytic phenotype by cytokine interactions.

Because tumor cell expression of endogenously-derived mutant *ras* epitopes may be a limiting event affecting CTL lytic efficiency, the isolation of CD8<sup>+</sup> T cells within the bulk population is performed. Such T cells may display a higher affinity for recognition of antigens that are present at extremely low-antigen densities. Furthermore, the interaction between higher affinity TCR with MHC/peptide may be of sufficient strength to obviate the need for Ag-independent

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interactions. As in the murine system, one approach will be based on conventional T cell cloning at limiting dilution and subsequent examination of lytic efficiency against target cells pulsed with titrating amounts of relevant peptide or tumor cells ( $\pm$ IFN- $\gamma$  pretreatment) harboring the *ras* oncogene. Clones are expanded on the appropriate mutant *ras* peptide (or defined peptide variant), with peptide dose gradually reduced over time during culture in an effort to derive and maintain the most Ag-sensitive clones. Similar studies can be conducted for the isolation of peptide-specific CD4<sup>+</sup> T cell clones, as determined by their proliferative capacity or cytokine secretion (e.g., IFN- $\gamma$ ) in response to titrating amounts of relevant peptide or exogenous sources of tumor-derived protein. The production and propagation of such oncogene-specific CD4<sup>+</sup> and/or CD8<sup>+</sup> T cell clones has direct implications for adoptive immunotherapy.

In addition, TCR- $\alpha\beta$ -chain usage of *ras* oncogene-specific T cell clones can be used to evaluate and correlate TCR phenotype (repertoire) with functional response. If predominant or restricted V $\alpha$  or V $\beta$  patterns are delineated, anti-TCR-specific MAb can be employed for more rapid cell isolation from original bulk or polyclonal populations of immune lymphocytes. The identification of such TCR- $\alpha\beta$  patterns may also lead to the development of molecular-based protocols for: (a) tracking the development of the T cell response following each vaccination cycle, as a quantitative measurement for monitoring Ag-specific immune status; and (b) the isolation and cloning of high affinity TCR as an experimental model to explore the capacity to transduce and functionally convert autologous naive lymphocytes into Ag-specific effector cells. The molecular technology for the high efficiency transduction of naive T cell populations with viral expression vectors encoding novel receptor molecules is known to those having skill in the art. The overall objectives of these studies enable the development and exploration of experimental cancer immunotherapy models that combine principles of both gene therapy and immunotherapy, which result in the generation of effector populations for adoptive transfer with modified and enhanced tumor-specific Ag recognition and targeting capabilities.

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